FISH BACTERIAL FLORA IDENTIFICATION VIA RAPID

CELLULAR FATTY ACID ANALYSIS

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Abstract

Seafood quality can be assessed by determining the bacterial load and flora composition, although classical taxonomic methods are time-consuming and subjective to interpretation bias. A two-prong approach was used to assess a commercially available microbial identification system: confirmation of known cultures and fish spoilage experiments to isolate unknowns for identification. Bacterial isolates from the Fishery Industrial Technology Center Culture Collection (FITCCC) and the American Type Culture Collection (ATCC) were used to test the identification ability of the Sherlock Microbial Identification System (MIS). Twelve ATCC and 21 FITCCC strains were identified to species with the exception of *Pseudomonas fluorescens* and *P. putida* which could not be distinguished by cellular fatty acid analysis. The bacterial flora changes that occurred in iced Alaska pink salmon (*Oncorhynchus gorbuscha*) were determined by the rapid method. Fresh fish contained up to 7 genera in which the aerobic plate counts (APC) was 3.04 log colony-forming units (CFU)/cm^2. As the fish spoiled, the APC increased to 6.60 log CFU/cm^2 and the flora was composed of *P. fluorescens/putida*, *Psychrobacter immobilis* and *Shewanella putrefaciens*. The Sherlock MIS rapidly and accurately identified seafood bacteria in fresh fish and can be used to monitor quality changes during iced storage of fish.

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Chapter 1: Review of the Bacteriology of Seafood Spoilage

1.1. Introduction

Deterioration in fish quality starts immediately after the fish is harvested. Fish undergoes microbial, biochemical as well as physical changes. Microbial spoilage is one of the most important factors in fish quality deterioration. Microorganisms present breakdown the biomolecules into volatile compounds which impart off-odors and off-flavors to the fish. Not all the microorganisms present on fish cause spoilage there are "specific spoilage organisms" which affect fish quality (Huss, 1995b). There are various methods being employed to isolate and identify these microorganisms. This can lead to better understanding of the microbial spoilage pattern and will help in devising intervention practices in the seafood industry to curb the microbial spoilage. Thus there would be a better chance for enhancing the shelf life of fish and to supply fresh fish to the consumers.

Pink salmon is one of the major fisheries in Alaska accounting for about 1.2 MMT (ADFG, 2006). Pink salmon is harvested either through drift or set gill netting, purse seining, trolling or beach seines in the Alaskan waters. Most of the pink salmon catches are turned to the fish processing industries located along the shore of Alaska. These industries process the salmon into cans and fillets (frozen or vacuum packaged). One of the major hurdles for this industry is the seasonality of the catch which gives the processors a very short time frame for utilizing and converting the high volume catch into various value added products. Taking into consideration the fisheries in Alaska, it takes almost 1-2 days for the salmon to reach from the harvest ground to the processor and additional 1 day for processing the fish. Time elapsed from harvest to processing may vary depending on location and the type of harvest. This influences the quality of the product delivered to the processors. At times, when substandard fish is received by processors, it may either be discarded or sent to a reduction plant for the manufacturing of fish meal and fish oil. During the period from harvest to delivery to the processor, the fish are stored in chilled sea water and later they are stored in ice. In this period there is a

good chance that the fish may undergo quality deterioration leading to a low quality final product and reduced shelf life.

This review focuses on the biochemical and microbial spoilage of fish, various fish spoilage bacteria, their spoilage patterns, rapid method to identify the microorganisms with an emphasis on the bacterial fatty acid based identification method.

1.1.1. Spoilage in seafood

Fish is a highly perishable commodity. Its spoilage can be attributed to the aquatic habitat it comes from, high moisture content, large amounts of non-protein nitrogen and the microorganisms associated with the fish. The spoilage process starts as soon as the fish is taken out of water, but with proper post-harvest handling the spoilage process can be retarded or controlled. Fish spoilage can be divided into physical, chemical and microbial spoilage.

1.1.1.1. Physical spoilage

Adenosine triphosphate (ATP) is used as an energy source when fish muscle contracts to form the actomyosin complex. When the fish is harvested, ATP is rapidly degraded converting the soft and elastic muscle into a tough, inflexible texture; this is the onset of *rigor mortis* in fish (Huss, 1995b).

When the fish are caught with gill nets or long lines, the fish struggle and die leading to a faster utilization of ATP and a faster onset of rigor. Hence it is beneficial to end the struggle of the fish as soon as possible which allows slower onset of rigor (Fletcher et al., 2003). Various methods have been devised for killing the fish. One of the methods commonly used is a blow in the head. Some researchers have used carbon dioxide as anesthetizer for king salmon (Fletcher et al., 2003) and arctic char (Jittinanadana et al., 2005) but the results obtained did not show any advantage over the traditional method.

After the fish undergoes rigor, the fish muscle softens again and enters the post-rigor phase. It is desirable to keep the rigor fish in chilled condition until rigor resolves which can otherwise lead to the breaking of the myocommata causing gaping of the fillets (Huss, 1995b).

In addition to the rigor mortis conditions, bacteria on skin play an important role in fish tissue damage. Skin provides an exposed surface area for the attachment of bacterial population ranging from 10^2-10^5 per sq. cm (Shewan, 1962) out of which a limited number of bacteria, chiefly proteolytic stains (Gill and Penney, 1977) invade the fish muscle (Murray and Shewan, 1979). Ryder et al. (1993) observed that the skin aerobic plate counts increased to 10^7 cfu per sq. cm. while the flesh had only 10^5 cfu per sq.cm. at the end of 11 days of hoki on ice. The increase in surface bacterial load with a corresponding increase in off-odors and flavors is probably a consequence of bacterial enzymes diffusing into the flesh and nutrients diffusing to the outside (Ruskol and Bendsen, 1992).

1.1.1.2. Biochemical spoilage

Fish is composed of protein (salt soluble and water soluble fractions), lipids, enzymes and non-protein nitrogen fractions which readily decomposed after death (Shahidi, 1994). Bacterial deterioration of these chemicals affects the quality of fresh fish during ice storage. Hence it's important to make a systematic assessment of the spoilage pattern and potential of these compounds.

1.1.1.2.1. Adenosine triphosphate degradation

Immediately after fish death, ATP is phosphorylated into its decomposition products. ATP is converted to adenosine diphosphate (ADP) which is converted to adenosine monophosphate (AMP), then to inosine monophosphate (IMP). IMP is sequentially converted to inosine and reduced to hypoxanthine, xanthine and other secondary

metabolites (Howgate, 2006). The decomposition of ATP to IMP takes place through the autolytic enzymes while the breakdown of inosine to hypoxanthine occurs through microbial action due to the action of bacterial nucleoside phosphorylase (Massa et al., 2005). As ATP degradation is a stepwise process, it can be used to determine the fish freshness. Based on the ATP degradation products, a measurement called K value has been developed and is the ratio of inosine and hypoxanthine to ATP, ADP, AMP and IMP. The lower the K value, the higher is the freshness of the fish (Saito et al., 1959). Fish caught in different seasons, different locations, stored in different conditions (MAP or aerobic storage) and physiological state during capture have shown to have a different ATP degradation rates (Boyle et al., 1991; Greene and Bernatt-Byrne, 1990; Hattula et al., 1993). *Pseudomonas fluorescens* and *Proteus vulgaris* isolated from spoiling cod produce purine nucleoside phosphorylase and degrade inosine to hypoxanthine (Surette et al., 1988, 1990).

1.1.1.2.2. Proteins

Fish protein fraction is composed of sarcoplasmic protein, structural protein and connective tissue protein (Huss, 1995a). Denaturation of protein during ice storage results into a decreased solubility (Dileep et al., 2005), increased hydrophobicity and oxidation of sulfhydryl group (Benjakul et al., 1997) of actomyosin. Enzymatic hydrolysis of proteins is caused by proteases which break the peptide bonds (Benjakul et al., 1997). Fish proteases can be classified according to the mode of their catalysis into serine proteases (trypsins and chymotrypsin), aspartyl proteases (pepsins and chymosin), cysteine or thiol proteases (cathepsins) and metalloproteases (collagenases and calpains) (Haard and Simpson, 1994). These enzymes have been reported to cause mushiness in crayfish (Kim et al., 1996), induce modori (gel weakening) in white croaker (Ohkubo et al., 2005), extensive gaping (Ando et al., 2002) and post-mortem tenderization (Kubota et al., 2001).

Along with the endogenous fish proteases, bacteria present on fish have been reported to cause proteolysis resulting in the production of low molecular weight compounds giving off-flavor and odors (Fraser and Sumar, 1998). Bacterial proteases, exopeptidases and endopeptidases catalyze protein hydrolysis resulting into peptides and amino-acids (Hase and Finkelstein, 1993). Proteolysis at chill storage temperature is due to the ability of psychrotrophic bacteria to produce extracellular proteases that are active at low temperatures. Psychrotrophic *Pseudomonas* strains isolated from fish produce proteolytic activity (Hoshino et al., 1997) and utilize amino acids (Zachariah and Liston, 1973) at temperatures as low as 0°C, hence can cause spoilage in fish held on ice. *Pseudomonas marinoglutinosa* and *Aeromonas* spp. are able to produce proteases at 0-2°C and actively degrade actomyosin (Venugopal et al., 1983). Maximum production of proteolytic enzymes at 5°C compared to higher temperature by one marine *Pseudomonas* (Kato et al., 1974) can be seen as a compensatory effect for lower enzymatic activity (Peterson and Gunderson, 1960). *Pseudoalteromonas* isolated from intestinal tract of hake was demonstrated to hydrolyze 84% of fish muscle protein at 7°C after 8 h of incubation (Belchoir and Vacca, 2006). Castell and Greenough (1957) showed that the some of the proteolytic *Pseudomonas* did not produce odor or produced mild musty or sour musty odors while those identified as non-proteolytic produced sweet, fruity, oniony and potato-like odors. Similarly, Kazanas (1968) found that 45 of 474 proteolytic isolates (comprising *Flavobacterium*, *Bacillus*, *Micrococcus* and *Pseudomonas*) showed activity on skim milk and not on raw perch juice while 10 of 474 isolates exhibited proteolytic activity only on raw perch juice. This indicated that certain organisms can hydrolyze milk proteins or fish proteins (Kazanas, 1968). Miller et al. (1973a) reported that the quantity of ethyl esters produced by *P. fragi* on homogenized milk and sterile muscle was different. On homogenized milk, the strain produced high amount of ethyl acetate, ethyl butyrate and ethyl hexanoate while the only major ester on sterile fish muscle was ethyl acetate along with minor quantities of ethyl butyrate, ethyl hexanoate and butanone were formed (Miller et al., 1973a). Hence spoilage ability of proteolytic bacteria can be better studied using the substrate related to the source of bacterial isolation.

Fish protein is in two fractions, the myofibrillar proteins and the water-soluble sarcoplasmic protein. Proteases of *P. marinoglutinosa* on actomyosin reduce the Mg^{++} dependent ATPase activity and hydrolyze protein (Venugopal et al., 1983). Dutson et al. (1971) found that the proteolytic activity of *P. fragi* on pig myofibrils lead to the complete disappearance of H zone and disruption of A band and some loss of dense material from Z line. Collagenase was detected from *Pseudomonas* sp. isolated from shrimp (Jabbar and Joishy, 1999). Belchoir and Vacca (2006) stated that the microbial proteases assist the hydrolysis of polypeptides into smaller peptides and amino acids resulting in easier absorption of these compounds inside the cell and that the protease production can be stimulated by presence of amino acids and peptides.

Although bacterial proteolytic activity is seen on myofibrillar protein, bacteria use free amino acids and nucleotides in the sarcoplasmic fraction before attacking the complex proteins during low temperature (Jay and Kontou, 1967). Lerke et al. (1967) observed that the protein fraction and the protein-free fraction of English sole juice showed different rates of bacterial spoilage. Although the bacteria (*Pseudomonas* Shewan groups I and III) grew well in both the fractions; the protein-free fraction spoiled faster without any off-odors or volatile reducing substances or TMA (Lerke et al., 1967). They observed that the rate of spoilage was higher when these two fractions were combined (Lerke et al., 1967). Proteolysis results in the formation of free amino acids (Makarios-Laham and Lee, 1993) which can be used for protein synthesis, generation of metabolic energy and recycling of co-factors (Christensen et al., 1999). These free amino acids can also be metabolized by bacteria to produce volatiles compounds giving off-odors to fish (Castell and Greenough 1959).

Decomposition of mono-amino monocarboxylic acids (e.g. glycine, alanine, etc.) by *P. fragi* form fruity odors while oniony odors can be formed from mono-amino (e.g. glycine, alanine, leucine, etc.), di-amino and heterocyclic acids (Castell and Greenough, 1959). Synergistic interaction involving ethyl acetate, ethyl butyrate and ethyl heaxanoate produced by *P. fragi* strains give a fruity odor in sterile fish muscle (Miller et al., 1973a).

The decomposition of branched chain amino acids (leucine, isoleucine and valine) by *Pseudomonas* leads to the formation of acetoacetate + acetyl-CoA, propionyl + acetyl-CoA and propionyl-CoA, respectively (Martin et al., 1973; Massey et al., 1976). The degradation products of leucine can further be metabolized by *Staphylococcus xylosus* to produce 3-methyl-1-butanol (Fukami et al., 2004). Addition of valine and leucine to aerobically fermenting fish mixtures by unidentified bacteria significantly increased the production of isobutyric + isohexanoic acids and isovaleric acid (Sanceda et al., 2001). Isoleucine, leucine and valine produce sweet, pelargonium-like aroma; methionine, produce sulfide-like odors while phenylalanine and tyrosine produce rose-like odors (Saisithi et al., 1966). Volatile aldehydes were reported to be from amino acids in fermenting fish sauce with *Bacillus* spp. as the major bacteria along with Coryneform, *Streptococcus*, *Micrococcus* and *Staphylococcus* (Saisithi et al., 1966. Proteolytic bacteria like *Aeromonas* and *Proteus* attack the muscle releasing tryptophan which is subsequently converted to indole by microbial activity. Non-proteolytic bacteria like *Flavobacterium* use the tryptophan released by proteolytic species and convert it to indole (Ashie et al., 1996). Chen and Levin (1974) observed that *Achromobacter* (*Psychrobacter* spp.) produced phenethyl alcohol from phenylalanine in the presence of ethanol with the release of NH_3. Ahamed and Matches (1983) demonstrated the ability of *Pseudomonas* to produce ethanol, isopropanol and propanol in fish tissue extract at 5°C. Lactic acid bacteria like *Lactobacillus delbrueckii* subsp. *delbrueckii* and *Lactococcus plantarum* isolated from ice stored maricultured turbot demonstrated proteolytic activity (Rodriguez et al., 2003). Tavaria et al. (2002) studied the volatile compounds produced by *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Enterococcus* using various amino acids (Table 1.1.).

Miller et al. (1973b) found that *P. perolens* (ATCC 10757) actively produced hydrogen sulfide and dimethyl trisulfide on sterile fish muscle. They explained the mechanism of the formation of sulfur compounds like methyl mercaptans, dimethyl disulfide, dimethyl trisulfide by bacteria during fish spoilage. Hydrogen sulfide is the only compound produced from cysteine by *Pseudomonas* while methionine degradation involves

oxidative deamination and subsequent demethiolation of methionine to give methyl mercaptan, α-oxobutyric acid and ammonia (Herbert and Shewan, 1976). Production of sulfur containing compounds from methanethiol derived from methionine, cystine or cysteine (Miller et al., 1973b) is shown in Figure 1.1.

Ringo et al. (1984) described the pathway for degradation of certain amino acids during trimethylamine oxide (TMAO) dependent anaerobic growth of *S. putrefaciens*. They found that cysteine and serine were completely oxidized during the conversion process to CO_2 with the release of hydrogen sulfide + ammonia and ammonia, respectively. Aspartate and glutamate were shown to be converted to oxaloacetate and α- keto-glutarate, respectively with release of alanine. Stenberg et al. (1984) showed that the uptake of cysteine, serine, glutamate, aspartate and lactate is stimulated by NaCl wherein Na^+ functions only in the transportation process.

Pseudomonas fluorescens, *P. putida*, *P. mendocina* and *Burkholderia* (*Pseudomonas*) *cepacia* isolated from hake produced amino acid decarboxylases and produce putrescine. *Shewanella putrefaciens* and *Aeromonas* spp. decarboxylated tyrosine and lysine to produce tyramine and cadaverine but in presence of TMAO these strains exhibited ornithine decarboxylase activity and produced putrescine (Baixas-Nogueras et al., 2003). Stenstrom and Molin (1990) showed that all *S. putrefaciens* strains isolated from seafood were ornithine decarboxylase positive while 8-33% of *Pseudomonas fluorescens/putida* showed lysine and ornithine decarboxylase activities. Similarly, Middlebrooks et al. (1988) reported the ability of *P. fluorescens/putida* isolated from Spanish mackerel to produce histidine decarboxylase at 15°C. However, *P. putida* lacks proteolytic activity and does not produce off-odors in fish and can be considered as a non-spoilage organism (Gennari and Dragotto, 1992).

1.1.1.2.3 Lipids

Changes in lipids occur through hydrolysis (Shewfelt et al., 1981) and oxidation mechanism (Haard, 1992). Lipid oxidation takes place by two pathways: non-enzymatic and enzymatic. The non-enzymatic lipid oxidation follows the classical free radical pathway through the initiation, propagation and termination steps (Choe and Min, 2006). In the initiation step, a free radical is formed from the lipid molecule which reacts with oxygen to produce a lipid peroxide radical. The peroxide radical reacts with a lipid molecule to form a hydroperoxide in the propagation step. In this step, the formation of another free radical keeps the degradation process continuous. This reaction terminates with the formation of products which cannot be utilized in the propagation reaction (Choe and Min, 2006). Hydroperoxides, the primary oxidation products, decompose into alkoxy radicals and then aldehydes, ketones acids, esters, alcohols and short-chain hydrocarbons (Choe and Min, 2006). The non-enzymatic lipid oxidation is enhanced by metal ions like iron, cobalt and copper as well as heme compounds (Haard, 1992). Enzymatic oxidation takes place by lipoxygenase enzyme initiating the lipid oxidation process by producing free radicals (German and Kinsella, 1985). Lipase activity depends on the fish species, the type of fish tissue (intestines, hepatopancreas and stomach) as well as fish size (Nayak et al., 2003).

Lipid oxidation can be assessed by a variety of chemical methods such as determination of conjugated dienes, peroxide value (primary oxidation products), anisidine value, thiobarbituric acid value and malonaldehyde content (secondary lipid oxidation products), and fluorescent products (interaction compounds produced from reaction between oxidized lipids and nucleophilic compounds) among others (Botta, 1995; Perez-Alonso et al., 2003). Lipid deterioration is also caused by the action of bacteria on the fish. Based on the substrate specificity, lipolytic enzymes from bacteria can be broadly divided into carboxylesterases (hydrolyze small ester containing molecules at least partly soluble in water) and true lipases (breakdown triacylglycerides) and phospholipases (breakdown phospholipids) (Arpingy and Jaeger, 1999). Lipases show positional

specificity towards triacylglycerides (Alford et al., 1961) based on which microorganisms can be divided into two groups: i) those which do not have any positional specificity such as *S. aureus*, *Corynebacterium acnes* and ii) those which can hydrolyze primary ester bonds, Sn-1 and -3 such as *P. fragi*, *P. fluorescens* (Jaeger et al., 1994). Lipases produced by organisms of the genus *Pseudomonas* can be divided into three groups based on their amino acid sequence (Jaeger et al., 1994). Group 1 consists of *P. aeruginosa* and *P. fragi*; group 2 of *Burkholderia* (*Pseudomonas*) *cepacia* and *B.* (*Pseudomonas*) *glumae* and group 3 of *P. fluorescens* (Rashid et al., 2001).

Triacylglycerol lipase has been isolated from *P. fluorescens* (Suguira et al., 1977) but the lipolytic ability is subjected to wide interspecies variation within genus *Pseudomonas* (Goldman and Rayman, 1952). *Pseudomonas fluorescens* can produce equal amount of enzyme at 0°C and 20°C (Alford and Elliott, 1960) while the lipase produced by *P. fragi* retain their activity at -7°C, -18°C and -29°C and exhibits an increased affinity to unsaturated fatty acids with decreasing temperature (Alford and Pierce, 1961). Conversely, *P. fragi* isolated from a dairy source was shown to lose 50% of lipase activity at 4°C and was lost completely at -15°C (Lu and Liska, 1969). Rey et al. (1969) observed that the enzyme production of *Pseudomonas* isolated from spoiling chicken increased at 5°C which was regarded as a compensatory effect to lower enzyme activity. The authors found that freezing did not affect the activity of extracellular lipases produced by *Pseudomonas* spp. Extracellular lipase secreted by microorganisms, breakdown the carboxyl ester bond in acylglycerol forming glycerol and the constituent fatty acids (Rashid et al., 2001). Glycerol, then is converted to dihydroxyacetone phosphate and finally to pyruvic acid through the glycolytic pathway while the fatty acid fraction can undergo β-oxidation to form acetyl CoA (Pelczar et al., 1993b). The free fatty acids can be oxidized to hydroperoxides which decompose into a variety of secondary oxidation products such as aldehydes, ketones, esters, carboxylic acids, alcohol and hydrocarbons (Choe and Min, 2006; Ross and Smith, 2006). Free fatty acids can also be converted to β-keto acids followed by decarboxylation to yield methyl ketones (Smith and Alford, 1969).

Although rare, some microorganisms like *Streptomyces* spp., *Pseudomonas ovalis* and *Micrococcus freundenreichii*, possess lipooxygenase enzymes which can lead to the formation of peroxides and their subsequent decomposition to the carbonyl compounds (Smith and Alford, 1969). The carbonyls like acetaldehyde, propionaldehyde, acetone and butanone can further be converted to their respective alcohols by *P. fragi*, *P. fluorescens*, and two other marine pseudomonads using alcohol dehydrogenase (Keenan et al., 1967). Similarly *P. ovalis*, *P. fragi* and *Pseudomonas* sp. completely remove 2, 4-dienals from rancid lard (Smith and Alford, 1969). Both, *P. fragi* and *P. aeruginosa* convert the carbonyl compounds on chicken adipose tissue to trace amounts of 2-butanone, 2-heaxanone and 2-octanone while *P. fluorescens* produce all these compounds in addition to acetone (Moerck and Ball, 1979). Generally considered as a non-spoiler, *P. putida* was shown to produce acetone, 2-butanone, methyl thioacetate, 2-nonanone in chicken muscle (Pittard et al., 1982). Methyl acetate, ethyl acetate, ethyl propionate and n-propyl acetate produced by *Moraxella* during spoilage gave ester notes to beef (Stutz et al., 1991).

Most of the previously published reports were experiments using lard and chicken as substrate which do not contain eicosapentaenoic acid and docosahexaenoic acids. The long-chain poly-unsaturated fatty acids are present at the Sn-2 position in the triacylglycerides which pose a sterical hindrance for the lipases with Sn-1 and -3 specificity to attack them (Jaeger et al., 1994). These fatty acids being unsaturated can be highly prone to oxidation by the non-enzymatic process (auto-oxidation) or enzymatic process (lipooxygenase and cyclooxygenase) to form lipid degradation products (Ashton, 2002). A 12-lipooxygenase isolated from gills has been shown to decompose arachidonic acid (20:4 ω6) and eicosapentaenoic acids (20:5 ω3) into short-chain volatiles (1-octen-3-ol, 2-octenal, 2-nonenal, 2-nonadienal, 1, 5-octadien-3-ol and 2, 5-octadien-1-ol) (Hsieh and Kinsella, 1989). In addition to these compounds, Josephson et al. (1984) described the degradation pathway of arachidonic and eicosapentaenoic acid to produce 1-octen-3-one, 1, 5-octadien-3-one, 3-hexen-1-ol, 2-hexenal, hexanal, 3, 6-nonadien-1-ol, 2, 6-

nonadienal, 3, 6-nonadienal and 2-octaen-1-ol. Choe and Min (2006) have described the oxidation products of oleic, linoleic and linolenic acid from edible oils (Table 1.2.). The oxidation products produced from the unsaturated fatty acids can be further decomposed by bacteria as discussed in this section. Thus the short-chain compounds derived from fish lipids lead to rancid smells.

1.1.1.2.4. Non-protein nitrogen fraction

The non-protein nitrogen fraction of fish comprises volatile bases (ammonia, mono-, di-, and trimethylamine), trimethylammonium bases (TMAO and betaines), guanidine derivatives (creatine and arginine), imidazole derivatives (histidine, carnosine and anserine), and other low-molecular weight compounds (urea, amino acids and purine derivatives) (Simidu, 1961).

Trimethylamine oxide (TMAO) is produced in marine teleosts from choline available in the diet which is converted into TMAO by microbial action in the intestines or tissue monooxygenase. The TMAO present in the fish diet is converted to TMA by intestinal microorganisms and then oxidized back to TMAO by the monooxygenase enzyme (Niizeki et al., 2003). After death, the TMAO in fish is reduced to TMA, then to dimethylamine (DMA) and formaldehyde by endogenous fish enzymes and also bacterial enzymes (Regenstein et al., 1982). The TMAO dimethylase activity was found in the myofibrillar fraction and the highest activity was observed at a pH range of 7.0 -7.5 (Kimura et al., 2000). The reduction of TMAO to TMA is due to bacterial action while DMA and formaldehyde is a result of endogenous enzymes (Regenstein et al., 1982). Estimation of TMA provides an important index for ascertaining the freshness of fish. Similarly an alternative method, total volatile base nitrogen (TVB-N) which measures ammonia, DMA and TMA, has been used for the same purpose. TMA and TVB-N have been used as a quality index for Mediterranean hake (Baixas-Nogueras et al., 2001), and aquacultured sea bass (Taliadourou et al., 2003), tropical wire-netting reef cod

(Jeyasekharan et al., 2005) and gilthead bream (Kyrana et al., 1997). Different threshold spoilage values exist for each fish, hence TMA and TVB-N threshold values are species dependent.

1.1.1.3. Microorganisms in fish spoilage

There has been a wealth of research conducted on ice and chilled storage of different species of fish from various climatic conditions. All studies have shown as ice storage period increases, the bacterial count increases. Bacterial population corresponds with an increase in TMA, TVB, hypoxanthine values and volatiles such as hydrogen sulfide, which leads to the sensory rejection of the fish (Massa et al., 2005; Kyrana et al., 1997; Sun et al., 2005; Taliadourou et al., 2003; Rodriguez et al., 2005). Fish microflora is dependent on the season of the capture, and the region of harvest. In northern waters, most of microorganisms found on fish are psychrophilic, growing well below 25°C (Shewan, 1962). Microflora of fresh pearl spot, a brackish water fish species from India, was reported to be composed of aerobic bacteria, H_2S producing bacteria (later identified as *S. putrefaciens*), *Brochothrix thermosphacta*, lactic acid bacteria, yeasts and molds (Lalitha et al., 2005). During 15 days of ice storage, the heterogeneous flora turned into a homogeneous microflora composed predominantly of *Pseudomonas* spp. followed by *S. putrefaciens*, *Aeromonas* spp. and *B. thermosphacta*. Similarly, Koutsoumanis and Nychas (1999) found *Pseudomonas* as a major portion of the population, followed by *S. putrefaciens, B. thermosphacta,* Enterobacteriaceae and *Photobacterium phosphoreum* (< 1%) in fresh Mediterranean boque. The microflora of sea salmon harvested from South Atlantic coastal waters had an initial microflora composed of *Pseudomonas* spp., *S. putrefaciens, Aeromonas* spp., *Moraxella* spp., *Acinetobacter* and lactose positive Enterobacteriaceae/*Vibrio* spp. (Hozbor et al., 2006). Fresh Mediterranean hake comprised of *Pseudomonas* and micrococci/staphylococci (Baixas-Nogueras, 2003). The initial microflora of pink salmon harvested from Alaskan waters comprising predominantly of *Flavobacterium* and *Moraxella*,but has a lower percentage of *Pseudomonas* (Himelbloom et al., 1994). The initial microflora of fish caught in different

places is different, although certain species like *Pseudomonas* are common. In fish caught in temperate and tropical waters, more gram positive bacteria are found than the fish from colder environments.

Although the microflora of fish during spoilage consists of a variety of organisms, there are "specific spoilage organisms (SSO)" which take part in the actual spoilage process. These organisms use proteins, lipids or other biochemical compounds present in fish and produce metabolites which are termed as microbial spoilage. *Shewanella putrefaciens* and *Pseudomonas* spp. have been identified as SSO in spoilage of fish under iced storage conditions (Gram and Huss, 1996).

1.1.1.3.1. Pseudomonas spp.

Organisms belonging to the genus *Pseudomonas* are aerobic, gram-negative, catalase- and oxidase-positive, motile rods possessing one or several flagella (Palleroni, 1984). The taxonomic definition of *Pseudomonas* given by Migula in 1894 was broad and vague leading to the incorporation of large number of incompletely characterized bacteria (Anzai et al., 2000). Organisms like *Achromobacter putrefaciens* were transferred into the genus *Pseudomonas*, as *P. putrefaciens*, based on their rod-like morphology, motility and non-fermentative metabolism (Long and Hammer, 1941). Stanier et al. (1966) studied 175 phenotypic characteristics of 267 non-pathogenic strains and grouped *P. fluorescens* into seven biotypes (A-G) and *P. putida* into biotypes A and B. Palleroni et al. (1973) identified five RNA-DNA homology groups at the genus level (Groups I to V). RNA group I consisted of three subgroups, the fluorescent strains namely *P. aeruginosa, P. putida, P. fluorescens, P. chlororaphis* and *P. aureofaciens* were partitioned in the first subgroup, the second subgroup comprised of fluorescent plant pathogens namely *P. cichorii, P. syringae* and *P. viridiflava* while the third subgroup included non-fluorescent species namely *P. stutzeri, P. mendocina, P. alcaligenes* and *P. pseudoalcaligenes*. The RNA group II consisted of plant and animal pathogens namely, *P. mallei, P. pseudomallei, P. caryophylli, P. cepacia, P. gadioli, P. pickettii* and

P. solanacearum. The third RNA group was composed of *P. acidovorans, P. testosterone, P. delafieldii, P. facilis, P. saccharophila, P. pseudoflava* and *P. palleroni*. The RNA group 4 had only two species, *P. diminuta* and *P. vesicularis* while RNA group 5 consisted of *P. maltophilia* and *Xanthomonas* spp. (Palleroni et al., 1973). Further research reclassified the organisms belonging to these RNA groups. The organisms belonging to RNA group II have been transferred to the newly formed genera like *Burkholderia* and *Ralstonia* (Yabuuchi et al., 1992; 1995) and some species belonging to RNA group III have been transferred to *Comamonas* (Tomaoka and Komagata, 1987), *Acidovorax* (Willems et al., 1990) and *Hydrogenophaga* (Willems et al., 1989). The RNA group IV species have been transferred to genus *Brevundimonas* (Segers et al., 1994), while *P. maltophilia* was transferred to genus *Xanthomonas* and further included in a new genus and renamed as *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). Only RNA group I comprise species belonging to genus *Pseudomonas* (Anzai et al., 2000).

1.1.1.3.2. Shewanella putrefaciens

The bacteria classified as *P. putrefaciens* (Long and Hammer, 1941) were later classified as *Pseudomonas* type IV (Shewan et al., 1960). Lee et al. (1977) renamed the organism as *Alteromonas putrefaciens* due to its low G + C content compared to *Pseudomonas*. MacDonell and Colwell (1985) separated this organism from *Alteromonas* and created a new genus *Shewanella*. Named after a noted fishery bacteriologist, Dr. James Shewan the taxonomic position of *S. putrefaciens*, summarized by Ziemke et al. (1998) and Vogel et al. (2005), belongs to the subdivision of gamma proteobacteria, order Alteromonadales, family Alteromonadaceae and genus *Shewanella*. Ivanov et al., (2004) elevated the genus to family level as Shewanellaceae. The major biochemical differences between *Pseudomonas*, *Alteromonas* and *Shewanella* are shown in Table 3.1.

Shewanella putrefaciens is one of the important SSO in fish (Huss, 1995b). Stenstrom and Molin (1990) tested several strains of *Pseudomonas* spp. and *S. putrefaciens* and

found *S. putrefaciens* strains formed four clusters and were different from rest of the *Pseudomonas* clusters. *Shewanella putrefaciens* produces a pink pigment, produce H_2S and ornithine decarboxylase and reduce TMAO (Stenstrom and Molin, 1990). *Shewanella putrefaciens* has high proteolytic activity in sterile fish muscle incubated at 1-2°C for 12 days (Miller et al., 1973c). The isolates actively produced methyl mercaptans, dimethyl disulfide, dimethyl trisulfide, H_2S and readily reduced TMAO to TMA. Hydrogen sulfide producing ability of *S. putrefaciens* is shown in Figure 1.1. Amino acids like cysteine and cystine, made available by fish proteolytic enzymes, are used by *S. putrefaciens* to produce H_2S (Lapin and Koburger, 1974). Baixas-Nogueras et al. (2003) demonstrated that *S. putrefaciens* utilized tyrosine and lysine to produce tyramine, cadaverine, and putrescine exhibiting ornithine decarboxylase activity. Jorgensen and Huss (1989) grouped *S. putrefaciens* isolated from spoiling cod into two groups based on their TMAO reduction ability, generation time at 25°C and salt tolerance. Ziemke et al. (1998) proposed that *S. putrefaciens* is a heterogeneous species comprising more than a single genomic group. They identified a new species, *S. baltica* which could produce N-acetyl-β-glucosaminidase and utilize a variety of carbohydrates as carbon sources. Vogel et al. (2005) characterized 518 strains of *Shewanella* spp. isolated from nine different fish caught from the Baltic Sea. They found that all the strains were able to decompose TMAO and produce H_2S but unable to ferment glucose. Based on the 16S-rRNA analysis, most of the species were identified as *S. baltica* and *S. alga* Vogel et al., 2005). Khashe and Janda (1998) used API-ZYM (Analytab Product Inc.) and fatty acid analysis to differentiate *S. alga* and *S. putrefaciens* from clinical isolates.

1.1.1.3.3. Psychrobacter immobilis

Gram-negative, oxidase-positive, coccobacilli which fail to produce acid from sugar termed as *Achromobacter* (Chen and Levin 1974) then reclassified under genus *Psychrobacter* as *P. immobilis* (Juni and Heym, 1986). The genus is closely related to *Acinetobacter* and *Moraxella* (Pacova et al., 2001) and has been isolated from skin of

whole and dressed sockeye, halibut, rock sole (skin-on), skinned fillets and trimmed fillets, skin and trimmed fillets of Pacific cod and Alaska pollock (Himelbloom et al., 1991) and spoiling pink salmon (Himelbloom et al., 1994). *Psychrobacter immobilis* differs from *Moraxella* in fermentation of glucose, mannose, arabinose, galactose and xylose in addition to the organisms lacking enzymes for hydrolyzing gelatin and starch (Preito et al., 1992; Pacova et al., 2001). Himelbloom et al. (1994) observed that *Moraxella* was a dominant species in the microflora of pink salmon stored on ice for eight days. Similarly, Gonzalez et al. (2000) reported that most of the bacteria associated with the spoilage of wild brown trout, pike and farmed rainbow trout from temperate water belonged to Moraxellaceae. Although these isolates exhibited lipolytic activity (Tween 20, Tween 80, tributyrin and egg yolk), their possible role in fish spoilage is questioned as none could produce typical spoilage compounds such as TMA and H_2S.

1.1.1.3.4. Interaction between microorganisms

Fish spoilage microflora is a dynamic mixture of bacteria and the change in microflora is affected by the substrate competition and antagonistic behavior of the organisms present (Gram, 1993). One such example is the ability of *Pseudomonas* spp. isolated from rhizospores of sugar beet has an ability to produce an antibiotic compound 2-4 diacetylphloroglucinol, (Haas and Keel, 2003). Similarly, Gram (1993) studied the potential of *Pseudomonas* strains and *Moraxella, Acinetobacter* and *Flavobacterium* strains isolated from spoiling and fresh fish for the ability to inhibit the growth of *Escherichia coli, S. putrefaciens, Aeromonas sobria, P. fluorescens, Listeria monocytogenes* and *S. aureus*. In addition *Pseudomonas* strains were observed to have ability to produce siderophore, an iron chelating compound, to inhibit the growth of *S. aureus* and *A. sobria* (Gram, 1996). These siderophore producing strains compete with *S. putrefaciens* for growth and may explain the dominance of fluorescent *Pseudomonas* spp. in bacterial fish spoilage and the low count of *S. putrefaciens* (Chai et al., 1968). Under iron limiting conditions, siderophores produced by *S. putrefaciens* were not able to

inhibit the growth of *E. coli, L. monocytogenes, S. aureus, A. sobria* and *P. fluorescens* (Gram, 1994). Baixas-Nogueras et al. (2003) suggested that *Aeromonas* spp. and *S. putrefaciens* exhibited a substrate competitive behavior in the utilization of tyrosine and lysine.

1.1.2. Rapid techniques for total bacterial count and specific bacteria

The classical techniques in seafood microbiology are very time consuming. The general procedure for estimating the total aerobic plate count is 24-72 h @ 25°C. In assessing fish product quality, it is beneficial to use rapid methods to estimate the total bacterial counts so that corrective action can be taken to avoid further microbiological deterioration of fish and reduction in the shelf life of the product. For accuracy and consistency of results, the methods used in characterization of bacteria should be universally applicable, require small and easily prepared samples, provide rapid and highly reproducible data, be capable of automation and handle high throughputs (Bull et al., 2000). The goal of rapid aerobic plate count detection is achieved by various methods developed by commercial laboratories.

1.1.2.1. Impedance technique for rapid APC detection

The impedance technique measures microbial metabolism in growth medium by changes in the ability of the medium to carry an electrical current (Sutton, 2005). Various commercial systems have been developed for the measurement of impedance, viz., Malthus (Malthus instruments), Rapid Automated Bacterial Impedance Technique (RABIT; Don Whitley Scientific), Bactometer (bioMérieaux) and BacTrac (Sy-Lab). The advantages of these methods based on impedance are (Harrigan, 1998) (i) ability to handle large number of samples simultaneously, (ii) respond faster to higher microbial loads leading to quicker analysis time, (iii) flag unsatisfactory samples that are automatically displayed. The batches from which such samples are taken can be marked

and stored for further analysis (Harrigan, 1998). This system depends on the electric frequency of the alternating current, capacitative component, and is temperature of operation. Hence a small change in temperature can lead to an increase in the capacitance and conductance leading to false results. Moreover, for using this system, a media which has been correctly buffered for greater conductance should be employed. Specially formulated media like, Total Count Brain Heart Infusion, total count Modified Plate Count Agar, Total Count General Purpose Media are used for the Bactometer (http://www.ourfood.com/General_bacteriology.html: accessed July 10, 2007). The RABIT system has a disadvantage of having high cost for the instrumentation (http://www.dwscientific.co.uk/rabit.php, 1999: accessed July 10, 2007).

These systems have been successfully tested for the detecting specific bacteria in foods such as *S. putrefaciens*, *Pseudomonas* spp. (Bagge et al., 2001; Salvat et al., 1997), and *Photobacteirium phosphoreum* (Dalgaard et al., 1997). None the less, the use of impedance technique is limited by the fact that (Gracias and McKillip, 2004):

1. Rapidity of the technique is inversely proportional to the initial count of bacteria
2. Require specialized costly equipment
3. Development of a reproducible standard curve which may be affected by variability in biochemical composition of foods and the variability introduced by stressed bacterial cells.

1.1.2.2. ATP bioluminescence

ATP bioluminescence technique has been widely used for total bacterial enumeration in the dairy industry and used in seafood microbiology. The technique works on the principle of production of luminescence by luciferin which is oxidized by luciferase enzyme in the presence of ATP (Harrigan, 1998). The amount of luminescence is compared with a standard luminescence produced by known amount of ATP (Harrigan, 1998). This method is fast, giving results in 30 sec. or less. Its main drawback of this

method is the possibility of non-microbial ATP which taking part in the luminescence reaction skewing the results (Vasavada, 1993; Himelbloom et al., 2003). Moreover, this method cannot detect low bacterial counts and hence conventional techniques need to be used (Murphy et al., 1998). Furthermore this technique gives information about the total bacterial count only and cannot identify individual organisms (Hofstra et al., 1994). Himelbloom et al. (2003) evaluated the use of ATP luminescence technique in salmon roe processing and found that the method correlated moderately ($r = 0.61$) with the conventional plate count technique and suggested that this technique can be used as a rapid quality control tool.

Similar to the above techniques there are numerous techniques being used in microbiology for rapid determination of bacterial content in foods such as the direct microscopic counts by membrane filtration (DEFT), flow cytometry, and turbidimetric methods. Some techniques only provide the estimate of total bacterial count and do not identify specific bacteria. However, all bacteria present on fish do not cause spoilage but only certain bacteria such as *S. putrefaciens* and *P. fluorescens* deteriorate the fish quality (Huss, 1995b). Therefore, estimation of the total bacterial count does not provide an accurate assessment of the microbial condition of the fish. The identification of individual bacteria is very important because it provides the needed information about the SSO presence in fish. Hence rapid methods to identify bacteria associated with seafood include carbon substrate utilization, nucleic acid sequencing and cellular fatty acids analysis.

1.1.2.3. Substrate utilization

Bacteria metabolize various carbon sources to derive energy. Each bacterial species utilizes specific carbon substrates from which they can be taxonomically identified. Various systems are available from bioMérieaux such as API 20E (for Enterobacteriaceae and species identification of gram-negative non-fermenting bacteria), API 20NE (for gram negative non-Enterobacteriaceae) and API 50CH (carbohydrate metabolism test),

API STAPH (for clinical staphylococci and micrococci) and MicroPlate from Biolog. These reactions are carried out in small cup shaped open capsules or microwell plates which contain specific substrates. When bacterial suspension is added to these wells or cupules, the resulting reaction produces a reaction specific color, precipitate or ring. Formation of these characters is termed to be a positive reaction which is given a score as in the API system or is just read as positive, intermediate or negative in the Biolog system. One of the main differences between the API and the Biolog system is the number of test substrates that differs from 20-30 to 95, respectively. The Biolog uses redox chemistry wherein during respiration, the bacterial cells reduce a tetrazolium dye added to the test substrate and produces purple color (O'Hara, 2005). In the API system, the scores obtained are converted into identification by using the APILAB software. In the Biolog system, the change in color in the microplates can be read by a spectrophotometer or the reaction results can be visually evaluated and manually entered. The tests are compared to the bacterial database calculating a similarity index (SIM) and then ranking of identifications by SIM values (O'Hara, 2005). The API system has been used for identifying gram-positive and gram-negative bacteria associated with the spoilage of horse mackerel (Rodriguez et al., 2005), chilled garfish (Dalgaard et al., 2006), farmed turbot (Rodriguez et al., 2003), smoked salmon (Himelbloom et al., 1996), whole Pacific herring and pink salmon fillets (Crapo and Himelbloom, 1999).
The Biolog system has been used in a the identification of food-borne pathogens (Odumeru et al., 1999), clinically important Enterobacteriaceae (Holmes et al., 1994), staphylococci and micrococci (Miller et al., 1993) environmental and ecological isolates (Konopka et al., 1998) and phytopathogenic *Corynebacterium* (Harris-Baldwin and Gudmestad, 1996). Very few studies have been published reporting the use of Biolog for the identification of bacteria related to seafood (Himelbloom et al., 2006 a,b). Al-Harbi and Uddin (2003) and Nedoluha and Westhoff (1997) used Biolog to identify bacteria associated with the gills and intestines of hybrid aquacultured tilapia and striped bass, respectively.

1.1.2.4. Nucleic acid based bacterial identification

The 16S ribosomal RNA (rRNA) is highly conserved among all organisms and is the best target to taxonomically differentiate between microorganisms. The procedure for sequencing the 16S gene involves a primer-specific reaction wherein the primer of the organism to be identified is added to the cellular debris and this primer pairs-up with the complementary rRNA, which is later amplified using the well described polymerase chain reaction (PCR) technique (Clayton et al., 1995). The amplified rRNA is purified, sequenced, and then matched with the gene database available on the internet or through commercial systems (Clayton et al., 1995). Molecular techniques have been mainly used for the identification of specific pathogens like *Salmonella, Clostridium botulinum* and *E. coli.* (Fung, 1994). These techniques are very important in determining the phylogenetic relationship between bacteria, thus helping in the process of establishing novel biotypes species, genus and families (Gillis et al. 2005).

Nucleic acid based identification technique has been used to identify bacteria related to spoiling freshwater fish (Gonzalez et al., 2000) such as *Psychrobacter, Acinetobacter, Moraxella, Chryseobacterium, Myroides odoratus, Flavobacterium* and *Empedobacterium* strains. Romero and Espejo (2002) found *Pseudoalteromonas* spp. with a 98% similarity when the 16S rRNA sequence was analyzed using the BLAST (Basic Local Alignment Search Tool). This led the researchers to conclude that *Pseudoalteromonas* spp. is the major spoilage causing organism in oysters (Romero and Espejo, 2002). Similarly, Vogel et al. (2005) used this technique for identifying the taxonomic position of the *S. baltica* associated with the spoilage of Danish marine fish.

1.1.2.5. Rapid technique using bacterial fatty acids

Fatty acids are a stable expression of the microbial genome and hence can be used as a tool to identify various microorganisms, which can be analyzed using gas chromatographic techniques. Microbial Identification Inc. (MIDI, Newark, DE)

developed a software named 'Sherlock Microbial Identification System' (Sherlock MIS) that works specifically with the Agilent Chemstation (Agilent Technologies, Wilmington, DE) for identification of fatty acids against company's database.

Further details about this technique are described in the next chapter (section 2.2). A particular microorganism in the library is compared against a database containing several to numerous strains.

The software compares the equivalent chain length (ECL) of each peak in the analysis with the expected ECL and names the peaks from C9:0 to C20:0 (Paisley, 2004). Based on the qualitative and quantitative comparison, multivariate statistics between the fatty acid profile of the test organism and library entry provides a descending list of similarity index (SMI) values from 1.00 – 0.00. A SMIof 0.500 or higher with a difference SMI of 0.100 between the first and second rank is considered to be a good comparison between the database and the previously unknown strains (Paisley, 2004). The Sherlock software has the capability of analyzing the fatty acid data statistically. The software can be used to create species dendrograms by employing principal component analysis from which clusters can be identified. These clusters represent the relatedness of the species and hence can be used to identify the taxonomic position of the closely related species (Paisley, 2004).

The Sherlock MIS has been used for identification of gram-negative non-fermenters (Osterhout et al., 1991), lactobacilli (Kankaanpaa et al., 2004), marine heterotrophs (Bertone et al., 1996), *Campylobacter* (Steele et al., 1998), *Desulfovibrio* (Dzierzewicz et al., 1996) and *Pediococcus* (Annous et al., 1999). Leonard et al. (1995) used the identification capability of Sherlock MIS with pulsed field gel electrophoresis for identifying methicillin-resistant *Staphylococcus aureus* and found excellent correlation.

There are few published studies on the use of Sherlock MIS in the identification of bacteria associated with seafood. This system was used for the identification of bacteria on the skin, gills, intestine and fillets of hybrid striped bass (Nedoluha and Westhoff,

1995 & Nedoluha et al., 2001), *Aeromonas* from fish and shrimp samples (Neyts et al., 2000) and histamine-producing bacterial strains isolated from canned anchovies (Kim et al., 2004).

Fatty acids have been used to characterize bacteria and to ascertain their taxonomic position (Kaneda, 1991). Whole cell fatty acid is a direct expression of cellular genome and is not affected by mutation, or acquisition or loss of plasmids. Moreover fatty acid analysis by GC (Gas Chromatography) is simple, fast and low cost. Other techniques like genomic fingerprinting and all PCR based variants, although effective, are not feasible for routine analysis because they are generally difficult to perform and expensive (Table 1.4.) (Komagata and Suzuki, 1987; Bertone et al., 1996).

The cellular fatty acid approach offers a similar level of specificity and sensitivity as genotypic methods (Welch, 1991). The procedure given by MIDI for extraction and analysis of cellular fatty acids are quick and straight forward. Depending on the skill of the analyst, 75 bacterial isolates can be used for extraction of fatty acids per day. High level of automation and a user friendly software coupled with GC helps in the quick identification of bacteria (5.8 min.). Although the initial cost of instrumentation is higher, the running cost is about $2.50 per sample (Sasser, 2006).

Along with taxonomic classification, bacterial fatty acid analysis has been used for epidemiological typing (Steele et al., 1998), studying thermal resistance for developing a cold pasteurization process (Annous et al., 1999), studying biosynthesis of *de novo* fatty acids (Fozo and Quivey, 2004), and the role of lactobacilli as regulators of PUFA (polyunsaturated fatty acids) absorption (Kankaanpaa et al., 2004). The study of fatty acids of food related bacteria under various stress conditions may elucidate the mechanisms that bacteria resist or adapt to improve the effectiveness of preservative factors (Beales, 2004).

1.1.3. Fatty acids location in cell membrane

Fatty acids are situated in the bacterial cell membrane. The outer membrane of Gram-negative bacteria is a lipid bilayer composed of lipopolysaccharide (LPS), phospholipids, and proteins (Figure 1.2.). The outer membrane is covalently anchored to the underlying peptidoglycan by means of Braun's lipoprotein (Pelczar et al., 1993a). The LPS are composed of lipid A, core polysaccharide, O antigen and phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acid attached (Figure 1.3.). All fatty acids in lipid A are saturated and some are directly attached to the NAG dimer which in turn are esterified to the 3-hydroxyl fatty acids (Todar, 2003). In Gram-negative bacteria about 25% of the outer membrane consists of glycerol-phospholipids (Seltmann and Holst, 2002).

1.1.3.1. Role of fatty acids in bacteria

Fatty acids play an important role in bacterial cell functions. Changes in environmental conditions such as temperature, pH, salinity, and growth phase affect the fluidity of the membrane. During stress conditions, the membrane fatty acid composition is altered to maintain fluidity (Aricha et al., 2004). The most common changes in fatty acid composition are alteration in the number of unsaturations, in the carbon-chain length and in the degree of carbon-chain branching (Aricha et al., 2004). The LPS, in the outer membrane forms the first permeability barrier of Gram-positive bacteria. LPS is permeable only to low molecular weight hydrophilic molecules (Todar, 2003). Bile salts, lysozymes, and many antimicrobials cannot penetrate the LPS which protects the cell from serum components and phagocytic cells (Todar, 2003).

Fatty acids act as activators of enzymes like protein kinase C and also modify proteins either by N-myristoylation, prenylation and palmitoylation. These modifications help in anchoring the protein in the cytosolic face of the membrane (Cooper, 2000). Lipids in bacteria also take part in the biological activity of the cells like substrate and enzyme

activation, also serving as a minor source of energy, or as an energy reservoir (Seltmann and Holst, 2002).

The gram-positive bacteria contain a combination of straight chain, unsaturated, iso- and anteiso- fatty acids while the proportion of hydroxyl fatty acids is very low (Paisley, 2004). Gram positive bacteria do not normally require, synthesize or contain sterols and polyunsaturated fatty acids (O'Leary and Wilkinson, 1988). Gram-positive organisms do not rely on cell wall lipids for defense against invasions and do not utilize intracellular lipids as an energy source (O'Leary and Wilkinson, 1988). Various species of *Arthrobacter* isolated from the water fern *Azolla* contain 85-97% of saturated iso- and anteiso-branched 13-19 carbon-chain fatty acids. Fatty acid content of ten species of *Bacillus* contain 15:0 anteiso, 17:0 anteiso, 15 iso, 16:0 iso and 17:0 iso (Kaneda, 1967). Lactic acid bacteria predominantly contain 25-64% of 18:1 fatty acids followed by 16:0 and 19:0 cyclopropane forms (Decallonne et al., 1991).

Gram-negative bacteria are characterized by the straight chain, unsaturated, hydroxyl, and cyclopropane fatty acids and can contain iso- and/or anteiso- fatty acids. *Salmonella* strains of epidemiological importance contain a large proportion of 16:1, 18:1 while 12:0, 15:0, 17:0 form the second most important group of fatty acids (Tas et al., 1988). *Helicobacter pylori*, contains 30% 19:0 cyclopropane fatty acid and 48% 14:0 (Scherer et al., 2003). The fatty acid composition of *Pseudomonas diminuta* contain 32% 16:0, 35% 18:1, and 13% 19:0 cyclo, while *Pseudomonas maltophilia* consists primarily of 34% 15:0 iso,11% 16:0 and 10% 14:0 (Moss et al., 1974). From spoiling fish *S. putrefaciens* contains 47% 16:1, and 13% 16:0 with traces of cyclopropane fatty acids and hydroxyl fatty acids while *P. phosphoreum* contains 56% 16:1, 13% 16:0, and 16% 18:1 fatty acids (Dalgaard, 1995).

1.1.3.2. Fatty acid as biomarkers

Bacterial fatty acids can be divided into two families based on their biosynthetic pathway: i) straight chain fatty acids, palmitic, stearic, hexadecanoic, octadecenoic, cyclopropanoic, 10-methyl hexadecenoic and 2- or 3-hydroxyl fatty acids and ii) branched chain fatty acids e.g., iso-, anteiso- omega alicyclic fatty acid, with or without a substitution by unsaturation or hydroxylation (Kaneda, 1991). Biomarkers can be used for characterizing and quantifying a specific microbial group or community and provide information such as microbial mass loading and microbial allergens concentration for health assessment (Lee et al., 2004).

The 3-hydroxy fatty acids are the unique structural components of endotoxin and can be used as biomarkers for gram-negative bacterial pathogens (Lee et al., 2004). The presence of 10:0 3-OH, 12:0 2-OH and 12:0 3-OH can be used as biomarkers to identify *P. aeruginosa* and *P. putida* from *P. acidovorans*, *P. testosteroni*, *P. stutzeri*, *P. alcaligenes* and *P. multivorans* (Moss et al., 1972). Quantitative difference of hydroxyl fatty acids and presence of 10:0 2-OH, 15:0 iso, 11:0 iso, 11:0 2-OH iso, 11:0 3-OH iso, and 13:0 3-OH iso can be used to identify *P. maltophilia* from *P. aeruginosa* and *P. putida* (Moss et al., 1973). *Pseudomonas* spp. can be divided into nine groups based on the existence of 3-hydroxy fatty acids (Oyaizu and Komagata, 1983). The presence of absence of 3-hydroxy fatty acids can also be correlated with rRNA groups I to IV of *Pseudomonas* (Vancanneyt et al., 1996). The rRNA group I, II, III and IV are characterized by the presence of 10:0 3-OH and 12:0 3-OH, 14:0 3-OH, 10:0 3-OH and 12:0 3-OH, respectively. Since, only rRNA group I has been retained in genus *Pseudomonas*, the biomarker fatty acids for the genus include 10:0 3-OH, 12:0 and 12:0 2-OH (Palleroni, 2005).

The biomarker fatty acids for genus *Shewanella* include 13:0 iso, 15:0 iso, 14:0, 15:0,16:1 ω7c and 18:1 ω9c (Bowman 2005). Qualitative and quantitative differentiation in the biomarker fatty acids can help in the identification of species (Table 1.5.). In addition to the regular mono-unsaturated fatty acids, *S. gelidimarina*, *S. benthica* and *S.*

hanedai (Leonardo et al., 1999), *S. marinintestina*, *S. schlegeliana* and *S. sairae* (Satomi et al., 2003) possess 20:5 ω3 fatty acid ranging from 11.7-23.1%. Satomi et al. (2003) identified the presence of 20:5 ω3 fatty acid in *S. pealeana* which absent for the same species by Leonardo et al. (1999). Both studies used the same strain of *S. pealeana* (Satomi et al., 2003) and the difference in the fatty acids was attributed to the different incubation temperature of 20°C (Satomi et al., 2003) and 28°C (Leonardo et al., 1999). Closely related genera like *Acinetobacter*, *Moraxella* and *Psychrobacter* can be distinguished by the presence of 12:0 2-OH (2%) in *Acinetobacter*, 3% 10:0 and 2% 17:0 iso in *Psychrobacter immobilis*, and 2-9% 10:0 and absence of 17:0 iso in *Moraxella* (Moss et al., 1988). 15:0 iso can be used as biomarkers for *Desulfovibrio gigas* while 17:1 (ω-7) iso, 15:1 (ω-7) iso and 19:1 (ω-7) iso can possibly be utilized as signature for lactate utilizing sulfur-reducing bacteria (Edlund et al., 1985). Based on the ratio of saturated and unsaturated fatty acids of soil and intestinal strain of *D. desulfuricans*, Dzierzewicz et al. (1996) suggested that that the intestinal *D. desulfuricans* should be represented by *D. desulfuricans* subsp. *intestinus*. High quantities of 16:1ω7c distinguishes *Legionnella geestiana* ATCC 49504 from other *Legionnella* spp. while low amounts of cyclic 17:0 forms an identifying character of *L. jordanis* (Diogo et al., 1999). High amounts of myristic acid, 19:0 cyclopropane fatty acid, β–hydroxyl palmitic and β–hydroxylstearic acids led to the taxonomic change of *Campylobacter pylori* to *Helicobacter pylori* (Goodwin et al., 1989; Geis et al., 1990). Fish pathogenic species *Pasteurella piscida*, has a similar fatty acid profile as *Vibrio anguillarum* and *A. salmonicida* suggesting close taxonomy (Romalde et al., 1995).

1.1.3.3. Stress related changes in cellular fatty acids

The cellular fatty acid types and concentration are liable to change as influenced by changes in culture condition, temperature, pH, pressure, salinity and other factors (Beales, 2004). Discrepancy in fatty acid profiles of the same strain of bacteria can be due to differences in the composition of culture media used in different investigations

(Dzierzewicz et al., 1996). Free polyunsaturated fatty acids (PUFA) from the growth medium can be incorporated into the bacterial cell inducing changes in the regulation of the amounts of linoleic acid and C20 to C22 PUFA (Kankaanpaa et al., 2004). Some bacterial strains may lose viability due to high uptake and low tolerance of fatty acids, hence fatty acid- free media is used (Scherer 2003).

Stress induced by the lowering growth temperature of *Psychrobacter* sp. isolated from Siberian permafrost, from 24°C to 4°C and -2.5°C reduced the proportion of saturated fatty acids from 72.5% to 13.7% to 60.2%, respectively and increased the degree of unsaturation from 3.7% to 75% and 21.2% (Ponder et al., 2005). Addition of 5% salt led to further increase in unsaturation from 87.2% to 90.6% and 74.3% respectively with decrease in temperatures from 24 °C to -2.5°C (Ponder et al., 2005). *Halomonas elongate* subjected to increased salt concentration, shifted the phospholipid pattern to more negatively charged species (phosphatidylglycerol and phosphatidylcholine) at the expense of more neutral species (phosphatidylethanolamine) suggesting an increase in structural integrity of the cell wall (Vreeland et al., 1984).

Increased degree of unsaturation increases the membrane fluidity facilitating the incorporation of lipophilic antimicrobial compounds like nisin and carvacrol in the membrane which results in slow growth of *L. monocytogenes* and *B. cereus* (Pol and Smid, 1999). Thus it can be deduced that the effect of nisin treatment can increase at low temperature and 5% salt concentration. An increase in temperature results in an increase in saturated fatty acids (Fouchard et al., 2005) and enables the acyl chain of fatty acids to form an optimal hydrophobic interaction eventually leading to a tightly packed, rigid membrane (Heipieper et al., 2003). In addition to an increase in saturation, psychrotrophic *Pseudomonas* and *Vibrio* have been shown to demonstrate a *cis* to *trans* isomerization of fatty acids when the temperature is increased to maintain membrane fluidity (Heipieper et al., 2003). Although saturated fatty acids have much higher phase transition temperature compared to *cis* unsaturated fatty acids (Heipieper et al., 2003), this response to stress is presented as rapid and emergency response (Loffeld and

Keweloh, 1996). *Pseudomonas* can secrete *cis-trans* isomerase enzyme through which they convert the *cis-* fatty acids into their *trans-* isomers thus increasing the membrane fluidity to counter the lipid-solidifying effect of cellular dehydration (Mortel and Halverson, 2004). Conversely, Gill and Suisted (1978) reported that an increase in temperature of psychrotrophic *P. fluorescens*, *Enterobacter* sp. and *Lactobacillus* sp. did not result in an increase in the saturated fatty acids. The fatty acid compositions were maintained but the 16:1 and 17:0 cyclopropane, 18:1 and 19:0 cyclopropane as well as any isoforms of the unsaturated compounds were not differentiated.

The thermal adaptation from 5°C to 25°C of 3 different *Aeromonas* spp. follows the similar pathway of changing unsaturated fatty acids to their saturated form but an increase to 42°C lead to an increase in iso 15:0 and iso 17:0 fatty acids which have a lower phase transition temperature than their saturated homologs (Chihib et al., 2005).

Starvation in bacteria results in the utilization of the *cis* monoenoic acid to produce *trans* monoenoic acids (which are not easily metabolized by bacteria) or modify the more volatile *cis* monoenoic acid to cyclopropyl derivatives to retain membrane fluidity (Guckert et al., 1986). Fouchard et al., (2005) reported that a change in the growth phase from exponential to stationary phase increased the cyclopropane fatty acid composition from 1% to 20%.

1.2. References

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Table 1.1. Amino acids and the respective volatile compounds produced by bacteria

Amino Acid	Volatile compounds
Alanine	Isopropylpropionate, propionic acid
Glutamine	Ketobutyric acid
Glycine	Acetic acid
Isoleucine	2-Methyl-1-butanol
Leucine	3-Methyl-1-butanol, 3-Methylbutyric acid
Methionine	Dimethyl trisulfide
Phenylalanine	Benzaldehyde, Acetophenone, Benzoic acid, Benzophenone
Phenylalanine/Tyrosine	Diphenylether
Serine	2-Ethoxyethanol, 3-Ethoxyethoxy-2-ethanol, Glycol
Threonine	2,3-Butanediol, 1,2-Propanediol
Tryptophan	Indole
Tyrosine	Phenol
Valine	2-Methyl-1-Propanol, 2-Methylpropionic acid, Isopropyl isobutyrate

(Adapted from Tavaria et al., 2002)

Table 1.2. Secondary oxidation products of fatty acid methyl ester by autooxidation

Class	Oleic acid	Linoleic acid	Linolenic acid
Aldehyde	Octanal	Pentanal	Propanal
	Nonanal	Hexanal	Butanal
	2-Decenal	2-octenal	2-Butenal
	Decanal	2-Nonenal	2-Pentenal
		2,4-Decadienal	2-Hexenal
			3,6-Nonadienal
			Decatrienal
Carboxylic acid	Methyl heptanoate	Methyl heptanoate	Methyl heptanoate
	Methyl octanoate	Methyl octanoate	Methyl octanoate
	Methyl 8-oxooctanoate	Methyl 8-oxooctanoate	Methyl nonanoate
	Methyl 9-oxononanoate	Methyl 9-oxononanoate	Methyl 9-oxononanoate
	Methyl 10-oxodecanoate	Methyl 10-oxodecanoate	Methyl 10-oxodecanoate
	Methyl 10-oxo-8-decenoate		
	Methyl 11-oxo-9-undecenoate		
Alcohol	1-Heptanol	1-Pentanol	
		1-Octene-3-ol	
Hydrocarbons	Heptane	Pentane	Ethane
	Octane		Pentane

(Adapted from: Choe and Min, 2006)

Table 1.3. Comparison between genus *Pseudomonas*, *Alteromonas* and *Shewanella*

Characteristics	*Pseudomonas*[1]	*Alteromonas*[2]	*Shewanella*[4]
Cell shape	Rods	Rods	Rods
Motility	Motile	Motile	Motile
Respiration	Aerobic	Aerobic	Facultatively Anaerobic
H_2S production	-	-	+
Acid production from glucose	+	-	-
Arginine dihydrolase	+	-	-
Gelatinase	+	+	+
Caseinase	+/-	+	+
Lipase	+/-	+	+/-
TMAO reduction	-	-[3]	+
Ornithine decarboxylase	-	-[3]	+
G + C content of DNA	58-69 mol%	44-48 mol%	34-58 mol%
Important fatty acids	10:0 3-OH, 12:0, 12:0 2-OH	16:0, 16:1 w7c, 17:1 ω8c, 18:1 ω7c	13:0 iso, 15:0 iso, 14:0, 15:0,16:1 ω7c, 18:1 ω9c

Ref: [1]Palleroni (2005), [2]Bowman and McMeekin (2005), [3]Trappen et al. (2004), [4]Bowman (2005)

Table 1.4. Advantages and disadvantages of using rapid microbiological techniques

	Polymerase Chain Reaction (PCR)	Substrate Utilization	ATP Bioluminescence
Advantages			
1	Low detection limit (10 cells/ml)	Alternative to conventional biochemical identification	Moderate detection limit (10^3 cells/ml)
2	*In situ* direct detection of bacteria	Miniature test-kits	*In situ* enumeration of bacteria
3	Rapid and sensitive identification and detection of bacteria	Simple procedure	Rapid method for enumeration (10-20 min)
Disadvantages			
1	DNA from non-viable organisms can give false positives	Involves isolation of bacteria; not *in situ* method	Only an enumeration technique; cannot identify bacteria
2	PCR inhibitors present in foods	Identification restricted to manufacturer's database	ATP in food interferes; requires sample pretreatment
3	Large-scale testing is time consuming; requires highly-skilled technicians	Physiological state of organism may affect substrate utilization and identification	Best suited for detecting contamination on food contact surfaces rather than foods

Table 1.5. Qualitative and quantitative comparison of selected fatty acid (%) within different *Shewanella* spp.

Fatty acid	*S. pealeana*	*S. putrefaciens*	*S. alga*	*S. woodyi*
12:0	4.0	0.1-2.6	0.1	4.1
12:0 3-OH	1.5	0	0	0
13:0 iso	13.8	1.0-3.6	0.5	10.0
13:0 iso 3-OH	3.2	0	0	0
14:0	5.6	1.5-5.9	0.7	4.6
15:0	2.5	1.4-7.5	4.0	4.0
15:0 iso	16.0	8.8-24.2	17.8	19.53
16:0	18.6	27.9-31.0	13.3	20.8
16:1 ω7c	20.1	25.2-31.4	16.3	0
16:1 ω9c	0	0.0-2.4	0	17.3
17:1 ω8c	2.8	2.7-3.7	14.7	0
18:1 ω9c	2.4	1.1-3.6	6.0	1.0

(Adapted from Leonardo et al., 1999)

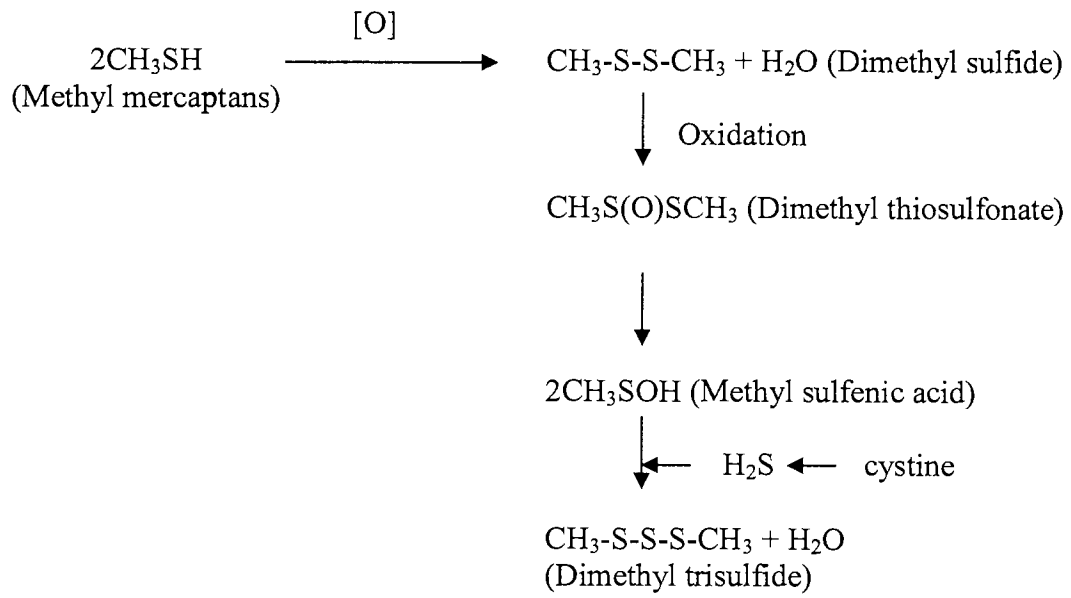

Figure 1.1. Formation of sulfur-containing compounds by bacteria
(Adapted from Miller et al., 1973b)

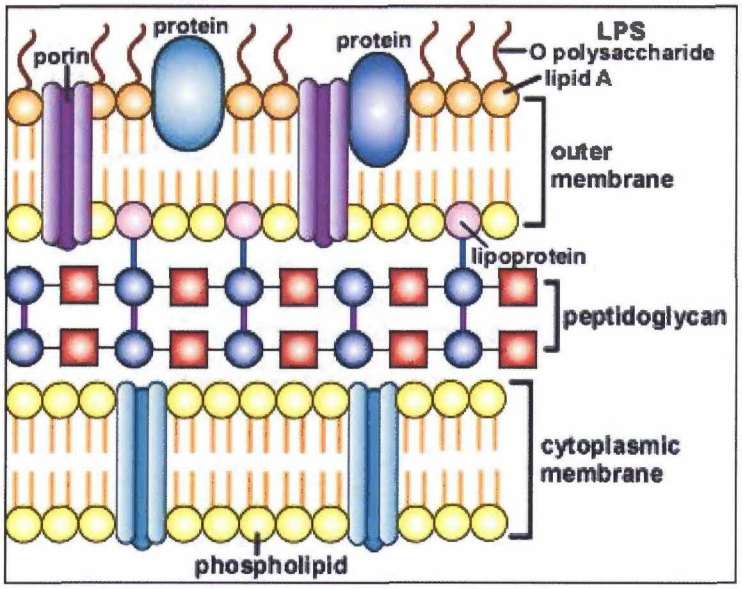

Figure 1.2. Structure of the cell surface of a Gram-negative bacterium (http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/diseases/gonococcus/u1fig10b.html : accessed July 11, 2007)

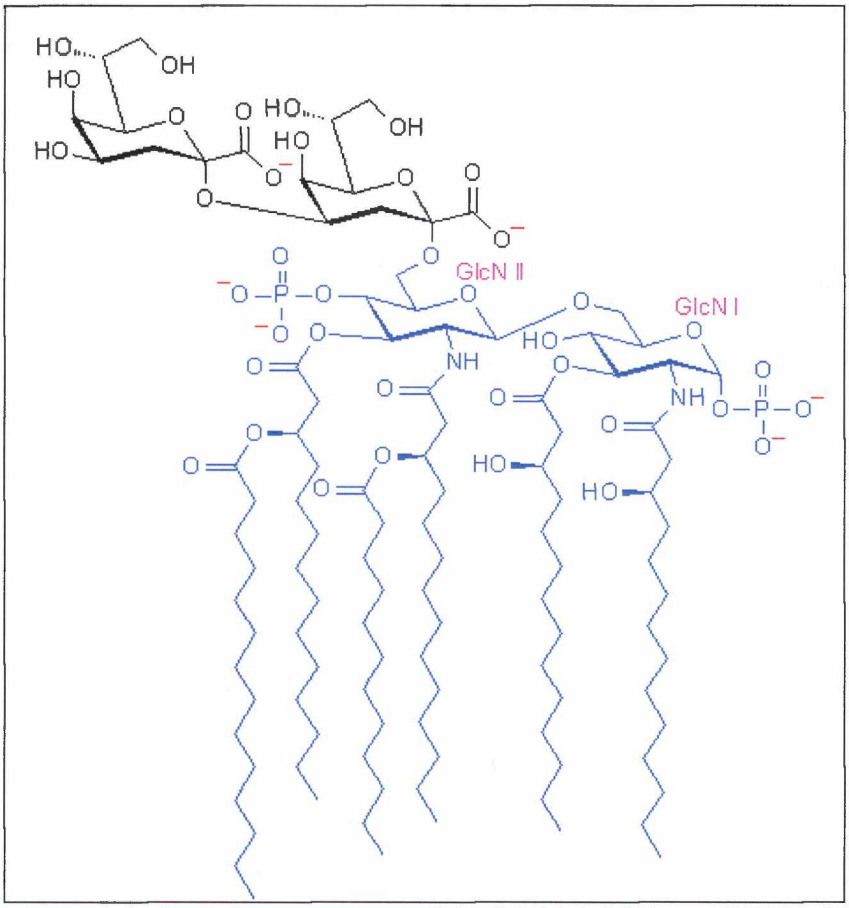

Figure 1.3. Complete structure of the Lipid A Moiety of LPS of *E. coli* (http://www.lipidlibrary.co.uk/Lipids/lipidA/image002.gif : accessed July 11, 2007)

Chapter 2: Whole-Cell Fatty Acid Analysis for Bacterial Identification Using the Sherlock MIS[1]

2.1. Abstract

The Sherlock MIS has been used as a rapid microbial identification tool in various fields of microbiology but the information is lacking on cellular fatty acids and identification ability of psychrotrophic bacteria from Alaskan seafood. Hence research was initiated to analyze the fatty acid content and identify bacteria associated with Alaskan seafood by Sherlock MIS. We also aimed at ascertaining the current taxonomic position of certain FITCCC strains.

Fatty acid methyl esters were extracted from the ATCC and FITCCC strains (n=33) and analyzed using standard protocols. The Sherlock MIS (4.5 version) was used to determine the identity of the bacteria based on their fatty acid profiles. Analysis was repeated 5-7 times for each isolate.

Gram-positive organisms had a high percentage of branched chain fatty acids (BFA) (*ca* 64-96%), except for *Streptococcus faecium*. Gram-negative, oxidase-negative isolates were composed of *ca* 38% to 69% of saturated fatty acids and *ca* 19% to 50% of unsaturated fatty acids. Hydroxy fatty acids were present in all the gram-negative, oxidase-positive isolates except *Pseudomonas fluorescens* 2A1, the BFA were <1% to 74%. Of the 33 isolates, 55% were identified at species level with similarity index (SMI) above 0.5 and within 0.1 SMI range of first rank. Strains of *P. fluorescens* and those belonging to Enterobacteriaceae were responsible for this lower percentage. The modern taxonomic position of some FITCCC strains was found to be *Shewanella putrefaciens*, *Psychrobacter immobilis* and *Myroides odoratus*. Sherlock MIS proved to be an effective and rapid technique in the identification of bacteria associated with seafood.

[1] Morey, A., Oliveira, A.C.M., Himelbloom, B.H., 2007. Whole-cell fatty acid analysis for the bacterial identification using Sherlock MIS. Prepared for submission in Journal of Microbiological Methods.

2.2. Introduction

Lipids compose almost 50% of the cytoplasmic membrane of the bacterial cell (Thornabene, 1985). Microbial cellular lipids can be categorized into an apolar fraction of phospholipids, glycolipids, neutral lipids and non-extractable lipids (Shaw, 1974). These lipid fractions play an important role as the structural and protective component as well as act as biologically active material for various cell functions and energy source or reservoirs (Seltmann and Holst, 2002). The diversity in the lipid species distribution among bacteria, easy and specific extraction and identification make cellular lipids the important chemotaxonomic biomarkers in bacterial systematics (Komagata and Suzuki, 1987).

The apolar lipid fraction comprises fatty acids which are bound to the cell membranes (e.g., phospholipids) or lipid A component of lipopolysaccharide in gram-negative bacteria, lipoteichoic acid in gram positive bacteria (Welch, 1991). According to Kaneda (1991), the bacterial fatty acids can be divided into straight chain (saturated, unsaturated, cyclopropane and hydroxylated fatty acids) and branched chain (saturated, unsaturated or hydroxylated iso-, anteiso- and ω-alicyclic fatty acids). These fatty acids can be biosynthesized (*de novo* and transformed) by bacteria through a multi-enzyme fatty acid synthase II pathway (Rock and Jackowski, 2002). A brief description of the biosynthesis of various fatty acids provides a background about the variations in fatty acid profile in bacteria. The developments in the fatty acid extraction methods and the identification ability of Sherlock Microbial Identification System (MIS) were reviewed in the previous chapter.

2.2.1. Fatty acid synthesis

The fatty acid biosynthesis is initiated with the formation of acetoacetyl-acyl carrier protein which undergoes a series of enzymatic reactions to give the principal final product of 16-18 carbon, saturated and monounsaturated fatty acids (Heath et al. 2001).

Saito et al. (1977) demonstrated that *Acholeplasma laidlawii* was able to synthesize C12-C18 fatty acids from short-chain primers of five carbons or less and stated that even- and odd-chain fatty acids can be synthesized from precursors like acetate and propionate or valerate.

The formation of unsaturated fatty acids occurs via the anaerobic pathway which introduces a double bond during fatty acid elongation or by aerobic pathway wherein direct oxygen-dependent desaturation of long-chain fatty acid produces *cis* double bond formation (Cronan, 2006). Fulco et al. (1964) reported that stearic and palmitic acids are aerobically desaturated to respective *cis*-Δ9 derivatives by *Corynebacterium diphtheriae* and *Micrococcus lysodeikticus*. They reported that *Bacillus megaterium* forms *cis*-Δ5 octadecenoic acid and *cis*-Δ5 hexadecenoic acid, not by direct desaturation of stearic and palmitic acid but by elongation of myristic and lauric acid to palmitic acid which are then desaturated. Okuyama et al. (1990) demonstrated a direct relationship of the increased level of 16:1 ω9t to the growth rate and attributed the increase to the *de novo* synthesis of the *trans* fatty acid in *Vibrio* sp. strain ABE-1. Diefenbach and Keweloh (1994) stated that isomerization of *cis* unsaturated fatty acids to *trans* form in *Pseudomonas putida* P8 strain occurs directly without *de novo* synthesis. Although the presence of polyunsaturated fatty acids in bacteria has been discussed by Russell and Nichols (1999), the biosynthetic mechanism is not clear.

Post-synthetic modification of palmitoleic (*cis*-9-hexadecenoic acid), *cis*-vaccenic (*cis*-11-octadecenoic acid) and oleic (*cis*-9-octadecenoic acid) already incorporated in the phospholipid fraction at the double bonds by cyclopropane fatty acid (CFA) synthase with S-adenosylmethionine (methylene donor) leads to the formation of *cis*-9-10-methylene hexadecenoic acid, *cis*-11-12-methylene octadecenoic acid (lactobacillic acid) and *cis*-9-10-methylene octadecenoic acid (dihydrosterculic acid), respectively (Magnuson et al., 1993; Schweizer, 1989). The biosynthesis and the factors controlling the cyclopropane fatty acids have been extensively reviewed by Grogan and Cronan (1997). They stated that the CFA formation occurs in the late exponential or early

stationary growth phase and the specific activity of CFA synthase is the primary determinant in CFA synthesis. They have also reported that the CFA synthesis is dependent on culture condition, genetic background and *in vivo* regulatory mechanism of the microorganism.

De novo synthesis of branched chain fatty acids can occur through the branched-chain fatty acid synthase mechanism which can use two different primer sources (Kaneda, 1991). The first primer source is the α-keto acids namely, α-keto-isocaproate, α-keto-β-methylvalerate and α-keto-isovalerate (which are related to valine, leucine and isoleucine) are used to synthesize iso-odd, anteiso-odd and iso-even fatty acids (Kaneda, 1991). The second primer source comprises the short-chain carboxylic acids like isobutyric, isovaleric and 2-methylbutyric acids which are required for growth and can be converted to branched chain fatty acids (Kaneda, 1991). Anteiso fatty acids are formed by using *S* isomers of 2-methylbutyric acid and 2-keto-3-methylvaleric acid (Kaneda, 1991). The ω-alicyclic fatty acids (ω-cyclohexyl-undecanoic, ω-cyclohexyltridecanoic and ω-cycloheptyl-α-hydroxyundecanoic acid) have been observed in *Bacillus acidocaldarius*, *B. cycloheptanicus*, thermoacidophilic *Bacillus* species and *Curtobacterium pusillum.* The formation of these fatty acids is thought to be from the cyclic-carboxylic acid as primers but the detailed pathway is unknown (Kaneda, 1991). As branched chain fatty acids have lower phase transition temperatures than the corresponding straight chain fatty acids, these can maintain appropriate membrane fluidity at lower temperature (Kaneda, 1991). Cropp et al. (2000) reported that in *Streptomyces avermitilis*, the branched-chain fatty acids contribute to the membrane fluidity but unsaturated fatty acids regulate the fluidity in response to growth temperature.

The formation of hydroxyl fatty acids has been explained through two pathways, one is the α– and β-type hydroxylation and the other is ω (terminal methyl group), ω-1, ω-2 or ω-3 type hydroxylation. The former types lead to the formation of 2- and 3-hydroxy fatty acids while the later system uses saturated, unsaturated fatty acids, alcohols and amides

to give a mixture of ω, ω-1, ω-2, ω-3 and ω-4 hydroxyl fatty acids. The β-hydroxy fatty acids are found in the lipid A fraction and contribute to the lipid A endotoxicity while poly-β-hydroxybutyrate serves as an energy source in bacteria (Schweizer, 1989).

2.2.2. Extraction methods

Abel et al. (1963) were the first to successfully demonstrate that gas chromatography (GC) can be used as a tool for bacterial fatty acid analysis. For the extraction and transesterification of fatty acids they used methanol and BCl_3 which were refluxed for 10 min on a hot plate stirrer (temperature not mentioned). The fatty acid methyl esters (FAME) were extracted with diethylether and analyzed on SE 30 (SE-30 refers to a linear end-capped polydimethylsiloxane gum produced by General Electric Company: http://www.freepatentsonline.com/4490314.html) silicone rubber as liquid phase to separate esters according to the boiling point with an oven temperature program of 125-300°C at 5.6°C/min. They achieved distinct fatty acid patterns for different microorganisms but were unable to get a clear-cut separation of unsaturated and saturated fatty acids. Brian and Gardner (1968) stated the existing method for separating cyclopropane fatty acids in bacterial fatty acid methyl esters by strong catalytic hydrogenation can lead to the formation of additional peaks in the FAME mixture. They proposed a three-step process to confirm the presence and identification of unsaturated and cyclopropane fatty acids: the FAME mixture is analyzed on the GC, hydrogenated to eliminate unsaturated fatty acids, re-analyzed and brominated to remove the cyclopropane fatty acids. Jantzen et al. (1974) achieved a good separation of iso- and anteiso-fatty acids by isothermal GC run at 160°C on 10% EGA (ethylene glycol adipate) column which allowed easy quantification under the peaks. The derivatization of FAME using trifluroacetic anhydride proved to greatly increase the peak sharpness for the hydroxyl fatty acids (Moss et al., 1973) and quantitative analysis (Mayberry 1980). Miller (1982) stated that although the acetylation method can restore the hydroxyl peak shape (no tailing), it causes a shift in the retention resulting in co-elution of the acetylated

hydroxyl acids and nonhydroxyl FAME. Miller (1982) suggested that the problem could be solved by a simple base wash of the organic extract to eliminate the need for derivatization. Moss and Nunez-Montiel (1982) suggested the use of fused-silica capillary column instead of packed column to achieve better separation and no peak tailing of short chain fatty acids (C2-C7) from bacteria. They advocated that the fused-silica capillary column could be used without breakage for extended periods and maintained efficiency. Orgambide et al. (1993) demonstrated that the use of HCl-CH_3OH, BF_3-CH_3OH and BCl_3-CH_3OH during *trans*-esterification process can lead to the formation of methoxylated fatty acid artifacts from 17:0 cyclopropane and 19:0 cyclopropane and recommended the alkaline hydrolysis FAME extraction procedure over acid hydrolysis. Mayberry and Lane (1993) devised a single tube extraction method for total fatty acids by sequential alkaline saponification, acid hydrolysis and esterification which enhanced the recovery of hydroxyl and cyclopropane fatty acids. The FAME extraction procedure was simplified by removing additional steps for each group of fatty acids. Mayberry and Lane (1993) addressed the issue of artifacts production during acid hydrolysis by avoiding BF_3 or BCl_3. They used 2M HCl after the saponification step and overlayed it with an appropriate alkane or aromatic solvent to partition the fatty acids, released during the saponification step, in the organic layer. Thus the contact of the cyclopropane fatty acids with the aqueous acid during hydrolysis was reduced (Mayberry and Lane, 1993). All the subsequent developments are mostly based on the changes in the time, temperature or substitution of certain chemicals with better ones.

Many researchers working on microbial fatty acids are using the standard protocol developed by Microbial ID Inc. (MIDI, Newark, DE). Use of a standard protocol can help in better comparison of fatty acid data generated by various researchers. As there was a growing implementation of fatty acid composition in identification of microorganisms, it was found necessary to use statistical tools for interpretation of the data and explore the relationship between the microorganisms. Researchers have applied various multivariate techniques and artificial neural networking (Noble et al., 2000) to gain a better understanding of the taxonomic position of test strains based on fatty acid profiles. A

computer assisted program can help the researchers analyze the results (Erola and Lehtonen, 1988).

2.2.3. Accuracy and reproducibility of Sherlock MIS

The only automated system available for the bacterial and yeast identification based on fatty acid profile analysis was introduced by MIDI in 1985. The highly computerized Sherlock Microbial Identification System (Sherlock MIS) identifies the fatty acids (C9-C20 chain length) and compares the calculated individual fatty acid percentages with its databases (fragmented into libraries). The comparison results in a list of possible microbial identities with the corresponding similarity index (SMI) calculated using multivariate statistical analysis. The Sherlock MIS program has a statistical program which allows quick comparison of fatty acid profile of the microorganisms (Welch, 1991; O'Hara, 2005).

Although the commercial systems for bacterial identification are time saving, these systems should be evaluated for their suitability to any particular laboratory (Miller, 1991). The MIDI system was also evaluated by researchers in diverse areas of microbiology and their experiences with the system have been published. Since the MIDI protocol and system produce equivalent and reproducible fatty acid profiles with a simple and time-saving procedure compared to the traditional, lengthy phospholipid fatty acid procedure (Haack et al., 1994), a number of publications report the use of MIDI protocol and the Sherlock MIS program for acquiring the fatty acid data. Osterhout et al. (1991) studied the ability to correctly identify the clinical bacteria as the first rank with SMI>0.5, first rank regardless of SMI and the reproducibility of the Sherlock MIS. They found that the system correctly identified 90% of the stains as the first choice while only 59% had SMI value above 0.5. They reported that among the 54 strains which were incorrectly identified, 37 unidentified bacteria belonged to *Acinetobacter*, *Moraxella*, *Alcaligenes* or *Pseudomonas pickettii*. A SMI range of 0.30-0.97 was obtained for *P. aeruginosa* ATCC 27853 in 100 replicate analysis and similar results were obtained for *Xanthomonas*

maltophilia (Osterhout et al., 1991). They concluded that the MIS system is an accurate, efficient and relatively rapid method for the gram-negative nonfermentative clinical strains. Birnbaum et al. (1994) demonstrated the successful use of MIS for its ease, predictive accuracy and reliability for the epidemiological typing of coagulase-negative staphylococci. Conversely, Steele et al. (1997) found that MIS could not be used for subgrouping of *Listeria monocytogenes* and verotoxigenic *E. coli* and concluded that the typing capability of MIS is species dependent. Haack et al. (1994) reported that from ten different soil bacterial isolates, two were not able to grow under MIDI specified conditions, four had SMI<0.3 and the remaining four had SMI>0.5. Nedoluha and Westhoff (1995) reasoned that the inability of MIS and other commercial systems to correctly identify the gram-negative rods associated with aquacultured fish was due to fact that the databases of these systems are composed mainly of clinical isolates and not environmental isolates. Brown and Leff (1996) suggests that the API 20E and NFT strips (bioMérieux Vitek, Inc., Hazelwood, MO) and Sherlock MIS needed further development before they can be successfully used for ecological identification of aquatic bacteria. They found a discrepancy in the identifications of strains, belonging to certain genera in Enterobacteriaceae, between API 20E and MIS. Tang et al. (1998) used the MIS for identification of clinically important fermenters and nonfermenters and identified 52% and 78% isolates to the species level. Odumeru et al. (1999) found that sensitivity (defined as the proportion of reference positive isolates correctly identified by the automated systems with an acceptable identification of >0.3 SMI and with 0.1 separation between first and second identification) and repeatability of MIS in identifying food-borne pathogens (six genera of forty isolates each) ranged from 47% to 90% and 30% to 90%, respectively, and were genus dependent.

2.2.4. Objectives

Based on the literature review in Chapter 1, fatty acids are species specific and the Sherlock MIS can be a bacterial identification system depending on the isolation niche

and species. The bacteria isolated from seafood in Alaska are mostly psychrotrophic and there has been no report on the fatty acid profile and the identification capability of Sherlock MIS of these bacteria. Hence, a research project was conducted by using Sherlock MIS (version 4.5) to analyze the fatty acid content and identify bacteria associated with seafood (Table 2.1). Several American Type Culture Collection (ATCC) strains were used as references. A wide variety of genera were selected to conduct a challenge test (Miller, 1991) of the system because Nedoluha and Westhoff (1995) had reported that the bacteria from fish could not be identified using the Sherlock MIS. Since the MIS uses fatty acid profiles to identify bacteria, the effect of repeated analysis on the variation in the fatty acid profile and its effect on the identification capability of MIS were assessed. There is a scarcity of literature on the fatty acid profiles of psychrotrophic seafood organisms. The building of an exclusive library to rapidly identify the spoilage organisms would be useful to determine seafood quality. Fishery Industrial Technology Center (FITC) maintains a frozen stock culture collection after isolating and identifying bacteria using classical biochemical and diagnostic tests and the API system. Hence, one objective was to verify the current taxonomic position of the test strains using MIS.

2.3. Material and methods

2.3.1. Bacterial isolates

Previously identified isolates from the Fishery Industrial Technology Center Culture Collection (FITCCC) and standard American Type Culture Collection (ATCC) isolates were used in the present study. The names of the isolates and source where isolated or obtained are in Table 2.1.

2.3.2. Characterization of microorganisms

All the isolates were subjected to gram reaction (Powers, 1995), catalase (Harrigan, 1998) and oxidase test (Kovacs, 1956). Morphological characteristics (shape and motility) of the selected microorganisms was observed under microscope (Zeiss, West Germany, Montagesaez T-UL, 467065-9914) by using the 40X and 100X oil immersion objectives.

2.3.3. Identification of bacteria using Sherlock Microbial Identification System (MIS)

The bacterial isolates stored under frozen condition (-80 °C) were thawed, revived and checked for purity by conducting basic biochemical characterization as given in section 2.3.2.. Pure cultures were streaked on TSBA plates (30 g of Trypticase soy broth (BBL, Sparks, MD) and 15 g agar (BBL) and incubated at 28°C for 24 h. Fatty acid methyl esters (FAME) were extracted in five replicates using the procedure given by Paisley (2004). The FAME were immediately analyzed on the GC model 6850 (Agilent Technologies, Wilmington, DE) which was coupled with a flame ionization detector (FID). The GC was equipped with an autosampler (Agilent 6850 Automatic sampler G2880A). A split injector was used with a split ratio of 40:1 and 2 μL of the extracted FAMES was injected for each analysis. The GC was fitted with Ultra 2 - 5% phenyl methyl silicone fused silica capillary column (25 m x 0.2 mm) Model 19091B-102E, Agilent Technologies, Wilmington, DE). Hydrogen gas (99.999% pure) was used as carrier gas at a flow of 1.4 mL/min (velocity = 0.6 m/s) with a pressure of 21.5 psi. The hydrogen gas used was generated in the laboratory using Agilent hydrogen generator (5182-3482). The oven temperature ramped from 170°C to 288°C at 28 °C/min and then to 310°C at 60°C/min. The total run time was 5.83 min. The FID was supplied with hydrogen (30 mL/min), air (350 mL/min) and nitrogen (99.999% pure) used as a makeup gas. The nitrogen was supplied by Air Products Inc. (Allentown, PA) while the air was generated in the laboratory using the Zero Air gas generator with compressor (ZA035A-ZA180A, Peak Scientific Instruments Ltd., Renfrew, Scotland). The Chemstation

enhanced integrator program (G.C. Chemstation rev. A. 10.02, 1757) was used to integrate the chromatogram peaks and electronically transferred to Sherlock MIS for library comparison with the TSBA50 aerobe database and report generation. The GC conditions were monitored strictly using the Sherlock MIS software.

The GC was calibrated using a calibration standard (No. 1300-AA) for Sherlock Rapid method which was obtained from MIDI. The standard contained FAME of straight chain saturated (C 9:0 to C20:0) and five hydroxyl fatty acids. The calibration standard was injected twice before the start of each analysis sequence as well as after every 11^{th} sample injection (Paisley, 2004). During all the calibration mixture analysis, the peak percent named for the standard was above 99% with the root mean square error below 0.0030.

2.3.4. Definitions

Known isolate or bacterium: The bacteria which were previously identified to genus or species level by classical microbiological techniques or API system.

Genus level identification (GLI): If the possible list of identifications given by Sherlock MIS consisted only a genus level matches, rather than species level for the known, <0.1 SMI unit of the first identification.

Species level identification (SLI): A species level identification of a replicate, of a particular known isolate, which is shown in the possible identification list irrespective of SMI from the first choice in the list provided by Sherlock MIS.

Mismatch: The replicate which has been identified to the SLI but is >0.1 SMI from the first rank in the possible identification list.

Not ID: The replicate of a known organism which did not show a correct identification to genus or species level irrespective of the SMI was named unidentified.

All ID >0.5 SMI within 0.1 range: The isolates which were consistently identified above 0.5 SMI and fell within the 0.1 SMI range of the first choice. Isolates with all identification >0.5 SMI and within 0.1 SMI range were marked as "100%". Although *Staphylococcus xylosus* had an SMI <0.5 it was included due to its consistency in SMI within replicate.

SF 1 (Summed Feature 1): The fatty acids, 15:1 iso OH and 13:0 3-OH which cannot be distinguished.

SF 2 (Summed Feature 2): The identification of a peak, tentatively designated as 12:0 aldehyde or unknown at retention time 10.947 min.

SF 3 (Summed Feature 3): The fatty acids, 16:1 ω6c and 16:1 ω7c, cannot be distinguished. Although the exact position of the double bond is not confirmed, it is clear that the peak of the fatty acid is unsaturated and was accounted in the calculation of unsaturated and total unsaturated fatty acids.

SF 4 (Summed Feature 4): The fatty acids, 10- Methyl 16:0 and 17:1 iso ω9c, cannot be distinguished.

SF 5 (Summed Feature 5): The fatty acids, 17:1 iso I or 17:1 anteiso B cannot be distinguished. The letters 'I' and 'B' denote that the position of the double bond is unknown.

SF 6. (Summed Feature 6): The fatty acids 18:0 anteiso and 18:2 ω6,9c cannot be distinguished.

SF 7 (Summed Feature 7): The fatty acids, 19:1 ω11c and 19:1 ω9c, cannot be distinguished.

SF 8 (Summed Feature 8): The fatty acids, 19:1 ω7c and 19:1 ω6c cannot be distinguished.

Accuracy: Calculated on the total number of SLI replicates to the total number of replicates.

Repeatable isolate: If all replicates of a particular isolate were correctly identified or all were identified to genus level.

Saturated or unsaturated straight chain fatty acid: The straight chain fatty acid without branching including cyclopropane fatty acids.

Branched chain fatty acids: The saturated, unsaturated and hydroxyl-fatty acids with iso- and anteiso- branching.

Total saturated fatty acids: The sum percentages of saturated straight chain, branched, hydroxyl and cyclopropane fatty acids.

Total unsaturated fatty acids: The sum percentages of unsaturated straight chain, branched, hydroxyl and SF 3 fatty acids.

2.3.5. Superimposing comparison charts

Comparison charts are generated by Sherlock MIS to show the fatty acid profile of the test bacterium with respect to the average library entry and range for respective fatty acids for a given microorganism. In this study the fatty acid profiles of the bacteria whose replicates had shown a deviation in identification for GLI, SLI, and/or Not ID were compared. The charts provide quick information about the deviations in the identification with respect to the changes in fatty acid percentages. Fatty acids are marked, with different colors for different replicates of a bacterium, which were outside the range. The reference comparison chart was chosen from among the replicates which showed the highest SMI value for the SLI. For generating these charts, CorelDraw (Designer 12, Ver. 12, Eden Prairie, MN) was used. Comparison charts of organisms correctly identified to the SLI in all the replicates were not shown. A comparison chart of *Psychrobacter*

immobilis ATCC 43116, wherein all the replicates were correctly identified is shown in Figure 2.9. For *Providencia alcalifaciens* 7F2, there was ambiguity in identification between API and MIS, therefore the average fatty acid profile of five replicates was superimposed on the RTSB50 (RTSB50 is the library of fatty acids of aerobic bacteria grown on TSBA for 24hrs at 28 °C) comparison chart of *P. alcalifaciens* and *P. rustigianii*.

2.4. Results and Discussion

2.4.1. Identification capability of Sherlock MIS

A total of 33 bacteria belonging to 18 genera (*Moraxella* sp 5E6 counted as *Psychrobacter* and *Alteromonas* sp 8C2 counted as *Shewanella*) comprising gram-negative and -positive strains were tested using the Sherlock MIS. Five or more replications were conducted to ascertain the ability of the MIS to identify the bacteria. All the FITCC isolates except *H. alvei* strains and *P. alcalifaciens*, were identified to GLI among all the replicates. All the FITCC isolates were identified to the SLI, one or more times within the replicates but there were 10 FITCCC isolates which were consistently identified above 0.5 SMI and were within the 0.1 SMI range of the first rank (Table 2.2). *Pseudomonas fluorescens* and various strains of Enterobacteriaceae did not show consistency in results within replications. Among the 12 ATCC isolates, all were identified to SLI, in one or more replication while 9 isolates were consistently identified above 0.5 SMI and within 0.1 SMI range. There were 5 instances where FITCCC isolates were not identified. The sensitivity of MIS to identify bacteria to the SLI was 73% of analysis conducted for the FITC strains and 92% for the ATCC strains (Table 2.4.). In all, the Sherlock MIS identified 54.6% of the total 33 isolates correctly to species level with SMI above 0.5 and the identification falling within 0.1 SMI range among all the replicates.

2.4.2. Gram positive bacteria

Gram-positive, catalase-positive rods (*Arthrobacter* and *Bacillus* spp.) and cocci (*Staphylococcus epidermidis, S. xylosus* and *Micrococcus varians*) and catalase-negative cocci (*Streptococcus faecium*) were analyzed for whole cell fatty acid composition.

2.4.2.1. Arthrobacter sp. 5B6

The *Arthrobacter* genus contains gram- positive, obligate aerobic rods or cocci with a guanine and cytosine (G+C) content of about 59-70 mol% DNA and these bacteria are isolated from soil and clinical specimens (Keddie et al., 1984; Funke et al., 1996).

2.4.2.1.1. Fatty acid profile

The fatty acid profile to *Arthrobacter* spp. 5B6 was primarily 15:0 anteiso-fatty acid, which comprised 80% of the total fatty acids while 20% was composed of saturated iso- and saturated fatty acids (Table 2.5). Funke et al. (1996) reported that major fatty acid content of various species belonging to *Arthrobacter* consisted of 34-75% of 15:0 anteiso, 3-29% of 15:0 iso, 3-15% of 16:0 iso, 2-11% of 16:0, and 6-25% of 17:0 anteiso fatty acids. The present data was comparable with the fatty acid profile of genus *Arthrobacter.*

2.4.2.1.2. Identification based on fatty acid profile

The bacterium isolated from Alaska pollock was presumptively identified from classical taxonomic testing as a species of *Arthrobacter. Arthrobacter* sp. 5B6 was identified as *A. aurescens* with an average SMI of 0.66 (SMI range of 0.60-0.87). It was identified as *Brevibacillus choshinensis* as the first rank which was closely (<0.1 SIM difference) followed by *A. aurescens* in two of five replicates. This variation in identifications can be

attributed to the slightly higher percentages of 14:0 iso and 15:0 iso fatty acids and a lower percentage of 15:0 anteiso fatty acid (Figure 2.1.). The variance of 15:0 anteiso fatty acid from the corresponding mean of the fatty acid library entry was deduced as the main cause of the change in identifications. However, MIDI suggests that a good match is considered when the SMI between the first and second rank ≥0.1. In the replicates which were identified as *A. aurescens* as the first rank, the second rank was *A. agilis* which had a SMI difference >0.1. In other replicates where *A. aurescens* was the second rank, it was separated from the first choice of *B. choshinensis* by <0.1 SMI unit. The fatty acid profile of *A. aurescens* reported by Funke et al. (1996) differed from the present study as it had 12% 17:0 anteiso and 3% 16:0 fatty acids. In conclusion, *A. aurescens* was considered to be the correct identification of *Arthrobacter* sp. 5B6 and that the identification results were reproducible.

2.4.2.2. Bacillus subtilis 5D1

The genus *Bacillus* comprises gram-positive, catalase-positive, aerobic endospore-forming rods which have meso-diaminopimelic acid in the cell wall (Claus and Berkeley, 1984). There has been significant changes in the position of various species in *Bacillus* since its inception by Cohn in 1872 and summarized by Fritz (2004). There were 31 species in 1989 which increased to 88 species and 2 subspecies in 2003 under *Bacillus* (Fritz, 2004). *B. subtilis* and *B. cereus* have been recognized as two important species in the genus (Fritz, 2004).

2.4.2.2.1. Fatty acid profile

The fatty acid profile of *B. subtilis* 5D1 contained a majority of anteiso-branched saturated (50%) and iso-branched saturated (37%) fatty acids while the saturated and the unsaturated straight chain fatty acids were present at 6% and 3%, respectively (Table

2.5.). This strain had 39% 15:0 anteiso-, 24% 15:0 iso-, 11% 17:0 anteiso, and 10% C17:0 iso-fatty acids and was similar the composition reported by Roberts et al. (1996). Kaneda (1977) reported *B. subtilis* strains were composed of 15:0 anteiso (33-40%), 15:0 iso (13-14%), 16:0 iso (11-9%), 17:0 anteiso (10%), and 17:0 iso (15-13%). Strain 5D1 had a low 2.3% of 16:0 iso fatty acid comparable to the results by Kaneda (1977). Branched-chain fatty acids especially from 14-15 occupy the Sn-2 position (second carbon atom in the triacylglyceride molecule) in a phosphatidylglycerol with a preference for anteiso- over iso-fatty acid (Kaneda, 1977). Additionally, branched chain anteiso-fatty acids have a melting point 25-35°C below the straight chain fatty acid's typical melting point and a decrease in culture temperature increases anteiso- fatty acids to maintain membrane fluidity (Kaneda, 1977).

2.4.2.2.2. Identification based on fatty acid profile

Strain 5D1 was identified correctly to the species level with a SMI of 0.83-0.94. For all replicates, *B. subtilis* was the first rank on the possible match list. There were three replicates having SMI >0.9 and *B. subtilis* as the only listing. The other two replicates had a SMI of 0.83 and 0.88; two to three identifications were separated from the first rank by <0.1 SMI unit. The comparison chart showed that all fatty acids of each replicate were within the range of the respective fatty acids used by the Sherlock MIS program for bacterial identification. According to Kaneda (1977), the genus *Bacillus* can be divided into two groups based on the dominance of either signature fatty acids 15:0 anteiso (*B. subtilis* group) or 15:0 iso (*B. cereus* group). From the present study, strain 5D1 was confirmed as *B. subtilis* and the identifications were reproducible.

2.4.2.3. Micrococcus varians 7F1

The Family Micrococcaceae includes *Micrococcus*, *Staphylococcus, Stomatococcus and Planococcus* (Schleifer and Kilpper-Baelz, 1984). *Micrococcus* (Kocur, 1984) is

described as small non-motile spherical, gram-positive, catalase- and oxidase-positive, aerobic cells with a G+C content of 64-75 mol% DNA. Additional characteristics of *Micrococcus*, composed of nine species, include high percentage of 15:0 iso and anteiso fatty acids, presence of high amounts of aliphatic hydrocarbon ranging from C22-C33, cardiolipin, phosphatidylglycerol and an unknown phospholipid (Kocur, 1984). Stackebrandt et al. (1995) divided the heterogeneous *Micrococcus* into four new genera based on phylogenetic and chemotaxonomic studies. Altering the broad description of the genus *Micrococcus* by Kocur (1984), Stackebrandt et al. (1995) restricted the genus to the organisms containing a G+C of 69-76 mol% DNA, phosphatidylglycerol, diphosphatidylglycerol and unknown ninhydrin-negative phospholipids and glycolipids; phosphatidylinositol may be present together with C27-C29 aliphatic hydrocarbons. Wieser et al. (2002) emended *Micrococcus* and divided *M. luteus* into three biovars based on polyphasic studies. Further development involved the reclassification of *M. luteus* ATCC 9341 as *Kocuria rhizophila* (Tang and Gillevet, 2003) and the reclassification of *K. varians* CCM 132 (Czech Collection of Microorganisms) as *K. craniphila* (Tvrzova et al., 2005).

2.4.2.3.1. Fatty acid profile

Micrococcus varians 7F1, being a slow grower was difficult to harvest for the standard required mass of 20-40 mg for fatty acid extraction and two to three plates were cultured simultaneously for obtaining sufficient cell mass. The fatty acid profile was dominated by branched 15:0 and 17:0 (Table 2.5.). The straight chain saturated and unsaturated fatty acids formed <4% of the total fatty acids. Branched 15:0 has been identified as a major fatty acid of *Micrococcus* (Wieser et al., 2002; Stackebrandt et al., 1995; Kocur, 1984). In addition to the major fatty acids, 13% of the fatty acids were 17:0 anteiso which may classify strain 7F1 as a species of *Kocuria* (Tang and Gillevet, 2003).

2.4.2.3.2. Identification based on fatty acid profile

Strain 7F1 isolated from smoked salmon was identified as *M. varians* by API Staph (600400 = good identification in 1996). In the present study, Sherlock MIS identified the isolate as *M. luteus*-GC subgroup B (includes ATCC 9341) with a SMI range of 0.69-0.86 with an average SMI of 0.77 (Table 2.2.). The four subgroups of *M. luteus* (A, B, C, D) have qualitative and quantitative differences. The individual fatty acids of all replicates were in the given range of the respective fatty acid entries for *M. luteus* subgroup B.

It can be concluded that the initial identification of *M. varians* 7F1 should be renamed as *M. luteus*. Further work needs to be carried out to ascertain the exact taxonomic position of this bacterium.

2.4.2.4. Staphylococcus epidermidis and *S. xylosus*

Spherical, gram-positive, catalase-positive, facultative anaerobic organisms forming clusters were classified under family Micrococcaceae and genus *Staphylococcus* (Kloos and Schleifer, 1984), then the genus was elevated to family Staphylococcaceae (Akatov et al., 1988). Staphylococci are ubiquitous in the environment and occupy a niche in warm-blooded animals and contaminated foods (Kloos and Schleifer, 1984). In addition to the foodborne disease-causing ability of these bacteria (Monsen et al. 1998), non-pathogenic species like *S. xylosus* are used as starter cultures in fermented meat products (Beck et al., 2004).

2.4.2.4.1. Fatty acid profile

Two species of *Staphylococcus*, *S. epidermidis* ATCC 14990 and *S. xylosus* 13A4, had similar fatty acids but there was a wide quantitative difference (Table 2.5.). Saturated straight- and branched-chain fatty acids were prominent in both strains. The FITC species

had a lower content of saturated straight- chain fatty acids (11%) while the ATCC species had 35% composed of 16:0, 18:0 and 20:0. The major fatty acids accounting for 58% of saturated iso- fatty acids in strain 13A4 included 27% 13:0 iso and 14% 15:0 iso. The total saturated anteiso- fatty acids for strain 13A4 (29%) and ATCC 14990 (35%) included 17% and 31% 15:0 anteiso- fatty acid, respectively. Strain 13A4 contained 13:0 anteiso (9%) while <1% was observed for *S. epidermidis* ATCC 14990.

Fatty acid profiles of *Staphylococcus* are characterized by the predominance of 15:0 iso and anteiso, 17:0 iso and anteiso (Spergser et al., 2003; Behme et al., 1996; Kotilainen et al., 1991). The fatty acid profile for *S. epidermidis* ATCC 14990 was similar but *S. xylosus* 13A4 had qualitative and quantitative differences with the fatty acid data in the references. As compared to the fatty acid profile of *S. xylosus* in the present study, Behme et al. (1996) observed that strains of *S. xylosus* contained high percentages of 15:0 iso, 15:0 anteiso, 17:0 anteiso, a very low content of 13:0 iso 14:0 iso and absence of 14:0 and 13:0 anteiso.

2.4.2.4.2. Identification based on fatty acid profiles

Among the five replicates for *S. epidermidis*, four were SLI- correct with average SMI of 0.68 while one replicate was identified with low confidence (SMI of 0.32) as *S. simulans* (Table 2.3.). When the fatty acid data of this particular replicate was superimposed on the comparison chart, it was observed that the percentage of 14:0, 16:0, and 18:0 were outside the ranges (Figure 2.2.). Certain fatty acids, such as 13:0 anteiso, 17:0 iso, and 19:0 iso and anteiso, were at the lower end of the ranges (Figure 2.2.). The wide variation in the fatty acid profile can be attributed to the difficulty in growing and harvesting the organism under the MIDI protocol. A slow growing bacterium, *S. epidermidis* ATCC 14990 required cultivation on seven TSBA plates to obtain 20-30 mg of cell mass for fatty acid extraction. Thus, the harvest made from the first and the slightly grown second streaks may alter the fatty acid profile of this replicate. Among the four replicates, two had SMI>0.7 and the other two had SMI of 0.58 and 0.67. The lower SMI can be

attributed to the higher percentage of 13:0 iso, 14:0, 16:0, lower 20:0, and absence of 19:0 and 19:0 anteiso. The next lowest SMI was due to the higher content of 14:0 and 16:0 which exceeded the percentage ranges for these fatty acids.

All five replicates of *S. xylosus* 13A4 were identified as *S. gallinarum* by Sherlock MIS with a SMI range of 0.37-0.42 with an average SMI of 0.39 (Table 2.2.). The manufacturer states that if the first match has a SMI of 0.3-0.5 and the first and second ranks are separated by >0.1 SMI unit then the match is good but the strain is atypical (Paisley, 2004). Accordingly, in the fatty acid profile, there was only one match in the possible identification list being *S. gallinarum.* Although *S. xylosus* 13A4 had most of the fatty acid composition qualitatively and quantitatively similar to the MIS, there were wide quantitative differences compared to the database entry. The 13:0 iso concentration differed by 25%, 13:0 anteiso differed by 8%, 15:0 iso differed by 10% and 15:0 anteiso differed by 22%. A similar explanation was given for the identification of *S. gallinarum.* Most of the fatty acids were within the library fatty acid range of *S. gallinarum* but 13:0 iso concentration differed by 14%, 13:0 anteiso differed by 4%, 15:0 anteiso differed by 14% and 17:0 iso differed by 5%. In addition, the presence of minor fatty acids 17:1 iso ω10c (<1%) and 19:0 anteiso (2%) might have been contributing factors toward the identification of *S. xylosus* as *S. gallinarum.* Stoakes et al. (1994) evaluated the reproducibility of Sherlock MIS for the identification of staphylococci including 15 strains of *S. xylosus* and found that the strains were SLI-correct.

Kotilainen et al. (1991) reported the fatty acid profiles of various *Staphylococcus* spp. are qualitatively similar but the quantitative differences can be used to distinguish this group. Birnbaum et al. (1994) analyzed the fatty acid profile using a statistical method of cluster analysis for various clinical coagulase-negative *Staphylococcus* spp. and could differentiate between various species and its source. Similar observations have been made by Leonard et al. (1995) on the methicillin-resistant *S. aureus*, a pathogen of high concern in medical establishments (Mulligan et al., 1993). They found a good correlation between the strain characterization by Sherlock MIS and the molecular biology tool,

pulse-field gel electrophoresis (PFGE), for differentiating species and strains. Meanwhile, Shutterworth et al. (1997) stated the fatty acid profiles of *S. caprae*, *S. capitis* and *S. haemolyticus* are similar. Stoakes et al. (1994) used the MIS and found 88% of 470 isolates were correctly identified while the incorrectly identified strains belonged to *S. haemolyticus, S. hominis, S. warneri, S. capitis,* and *S. saprophyticus.*

It can be concluded the fatty acid profiles for two *Staphylococcus* spp. could be differentiated. Based on the data and the library match, *S. xylosus* 13A4 should be considered for renaming as *S. gallinarum.* More research would have to be conducted to confirm the new taxonomy of this strain. This particular isolate can be termed as reproducible as all identifications of respective replicates were the same.

2.4.2.5. Streptococcus faecium 6A4

The genus *Streptococcus* is grouped into hemolytic streptococci, viridans streptococci, enterococci and lactic streptococci (Clancy, 1977). Schleifer and Kilpper-Baelz (1984) proposed the formation of genus *Enterococcus* and included *S. faecalis* and *S. faecium* belonging to Lancefield group D into the newly formed genus based on DNA relatedness.

2.4.2.5.1. Fatty acid profile

Streptococcus faecium 6A4 isolated from a commercial starter triculture was gram-positive, catalase-negative with ovoid cells. The fatty acid analysis indicated that *S. faecium* exhibited very limited diversity in fatty acid composition (Table 2.5.). The profile consisted of 14:0 to 18:0 and 19:0 cyclo ω8c saturated fatty acids and SF 3 (16:1 ω6/7c), 18:1 ω7c and 20:2 ω6, 9c unsaturated fatty acids. The major fatty acids comprised of 18:1 ω7c (48%), SF 3 (17%), 16:0 (17%), 19:0 cyclo ω8c (9%) and 14:0 (7%). Unsaturated fatty acids formed 66% of the fatty acid content of *S. faecium.* Amstein and Hartman (1973) used the fatty acid profiles for differentiation between four

species of enterococci obtained from human, animal and plant sources. Although they were unable to distinguish these enterococci, they were able to differentiate quantitatively between *S. faecalis*, *S. faecium*, *S. faecium* var. *durans* and *S. faecium* var. *casseliflavus*. The former three species showed minor quantitative differences while the latter species could be distinguished from the others based on very low or lack of 19:0 cyclo and presence of unknown fatty acids. Similar fatty acid profiles have been reported by Farrow et al. (1983). In spite of these similarities, there were large quantitative differences between the present study and the published studies.

2.4.2.5.2. Identification based on fatty acid profile

Although the total response was below 50,000 for all replicates which is ideally recommended in the MIDI manual, all replicates were identified as *E. faecium* (*S. faecium*) with an average SIM of 0.78 (Table 2.2.). The low total response is due to slow growth of *S. faecium* on TSBA leading to low amounts of cells during the harvesting step. All possible identifications in the listing consisted of *E. faecium* GC subgroups A and B. The RTSB50 library consists of four entries of *E. faecium* (GC subgroup A, B, C and D). Subgroups A, B and D possess similar fatty acid profiles but differ quantitatively while subgroup C additionally has 15:0, 11-methyl 18:1 ω7c, 20:2 ω6, 9c and 20:4 ω6, 9, 12, 15c (RTSB 50 library entry information). Taking into consideration the changes in taxonomy *S. faecium* 6A4 is confirmed as *E. faecium* and reproducibility of the MIS in identifying the isolate was shown.

2.4.3. Gram-negative oxidase-negative bacteria

2.4.3.1. Citrobacter freundii 4E5

Facultatively anaerobic, gram-negative, oxidase-negative, catalase-positive straight rods belonging to Enterobacteriaceae which can utilize citrate as a sole carbon source are

classified as genus *Citrobacter* (Sakazaki, 1984a). Bacteria belonging to this genus are associated with sewage, soil, water, food, human clinical specimen, aquacultured fish, and other veterinary sources (Toranzo et al., 1994; Sakazaki, 1984a). There are 11 species described under the genus including the type strain *C. freundii* (Frederiksen, 2005).

2.4.3.1.1. Fatty acid profile

The fatty acid profile for strain 4E5 showed 16:0 and SF 3 plus 18:1 ω7c as the major fatty acid contributing to 38% of total saturated and 50% to total unsaturated fatty acids, respectively (Table 2.6.). Cyclopropane fatty acids and other branched chain fatty acids formed a minor component of the fatty acid profile. MacTiger and O'Leary (1973) studied the fatty acid profile of three different strains of *C. freundii* cultured to the stationary phase and found that all strains consisted of >30% of total cyclopropane fatty acids, the total unsaturated acids ranged from 11% to 22% while the total saturated fatty acids accounted for 41-52%. These differences in fatty acids, when compared with strain 4E5 was probably due to the strain-specific differences, media used, growth phase and the extraction method used by MacTiger and O'Leary (1973). Toranzo et al. (1994) isolated *C. freundii* strains from fish (salmonids and non-salmonids), humans and veterinary sources had similar fatty acid profile but the salmonid strains could be differentiated from others based on significant differences in 16:0, 16:1 and 17:0 cyclopropane fatty acids. Strain 4E5 had a quantitatively different fatty acid profile than those studied by Toranzo et al. (1994). Different strains have different fatty acids and cellular fatty acid content can be effectively used to differentiate between *C. freundii* strains (Toranzo et al., 1994; MacTiger and O'Leary, 1973).

2.4.3.1.2. Identification based on fatty acids

Citrobacter freundii previously identified using API 20E was correctly identified in four of five replicates by the Sherlock MIS system with a average SIM of 0.65 (Table 2.2.). In the two replicates, the first match was *Erwinia chrysanthemi* which was separated from

C. freundii match by <0.1 SMI unit in two replicates but >0.1 SMI unit in the other two replicates. *Erwinia, Escherichia, Salmonella* and *Serratia* are the nearest genetically-related genera to *Citrobacter* (Frederiksen, 2005). Strain 4E5 was not identified for one replicate from any of the choices in the possible identification list. The manufacturers recommendation of using the difference of <0.1 SMI unit between the first and second ranks did not fit clearly. These discrepancies between the replicates can be explained by the closeness of the individual fatty acids with the corresponding fatty acid mean percentage and the percentage range used by Sherlock MIS library for that fatty acid in the comparison chart. Most of the fatty acids of all replicates were either near the mean or within the prescribed percentage range (Figure 2.3.). In one replicate, which was not identified as *C. freundii*, lower 16:0 (21%) and 18:1 ω7c (14%) and higher SF 2 (13%) and SF 3 (34%) than the percentage range of the corresponding fatty acid library entries for *C.* freundii were observed. A similar explanation was given for the difference of >0.1 SIM between first rank and *C. freundii*.

2.4.3.2. Enterobacter cloacae 12D5

Gram-positive, catalase-positive, oxidase-negative, facultatively anaerobic glucose-fermenting rods with G + C of 52-60 mol% DNA belonging to family Enterobacteriaceae are grouped into genus *Enterobacter* (Grimont and Grimont, 2005b). These bacteria are associated with natural environments, nosocomial infections and powdered breast milk substitutes.

2.4.3.2.1. Fatty acid profile

The total saturated (51%) and total unsaturated fatty acids (38%) formed the major fraction of the fatty acid profile of *E. cloacae* 12D5 (Table 2.6.). The important contributors to these fractions were 16:0 and 18:1 ω7c plus SF 3. Branched chain fatty acids were completely absent in all replicates.

2.4.3.2.2. Identification based on fatty acid profile

Enterobacter cloacae 12D5 was identified to the species level in three of five replicates but it was mismatched as *E. coli* at the first rank in the possible identification list (Table 2.2.). The difference between the first rank and *E. cloacae* was >0.1 SMI unit. All identifications as *E. cloacae* had a narrow SIM range (0.776-0.779) and the differences between fatty acids quantities were very subtle. The possible explanation for *E. cloacae* not being the first rank was the difference between the mean fatty acid percentage of the library entry and the replicates with respect to 17:0 cyclopropane (difference of about 3%), 18:1 ω7c (difference of about 1.6%) and SF 3 (difference of about 3%). The former two fatty acids were outside the percentage range of the library entry (Figure 2.4.). Similarly, the identifications of the other two replicates identified only to genus level can be explained by the higher percentage of 17:0 cyclopropane (10-11%) and lower percentage of 18:1 ω7c (20-21%) and SF 3 (12-14%). The percent differences between these fatty acids and the library entry means were about 6% for 17:0 cyclopropane, 3-4% for 18:1 ω7c and 7-10% for SF 3.

The identification capability of *E. cloacae* 12D5 was challenged by its misidentification as *E. coli*. Additional research will be needed to improve the reproducibility of this strain's identity.

2.4.3.3. Escherichia coli ATCC 11303

Genus *Escherichia* consists of gram-negative, oxidase-negative, facultatively anaerobic rods capable of fermenting most carbohydrates (Scheutz and Strockbine, 2005). The genus comprises *E. coli, E. blattae, E. fergusonii, E. hermannii* and *E. vulneris*, which can be differentiated based on phenotypic and genotypic characterization, occur in the intestines of humans, animals and insects (Scheutz and Strockbine, 2005).

2.4.3.3.1. Fatty acid profile

The major fatty acids of *E. coli* ATCC 11303 were 16:0, 17:0 cyclopropane, 18:1 ω7c, SF 2 and SF 3 (Table 2.6.) which were the major contributors to saturated (39%), cyclopropane (13%) and unsaturated (38%) fatty acids. Although the major fatty acids in *E. coli* are 14:0, 16:0, 16:1 and 18:1 (Subrahmanyam and Cronan, 1998), ATCC 11303 had about 6% of 14:0. This strain differs from pathogenic *E. coli*, such as biotype O157:H7, due to higher 16:0 and lower 17:0 and 19:0, and the absence of 14:0 3-OH and 18:1 ω9c in the former (Chiou et al., 2004). The fatty acids of 16:0, 16:1 and 18:1 are associated with the phospholipids while 12:0 and 14:0 3-OH is exclusively found in lipid A in the lipopolysaccharide (Subrahmanyam and Cronan, 1998). Among the replicates, the 17:0 cyclopropane content varied from 6 to 12% with a concomitant shift in SF 3 from 12 to 7%. This suggests a modification of the 16:1 fatty acid into 17:0 cyclopropane which is an indication of change in growth phase (Grogan and Cronan, 1997).

2.4.3.3.2. Identification based on fatty acid profile

The ATCC 11303 strain was identified correctly to species level (Table 2.3) in all five replicates as either the first rank or the second rank within 0.1 SMI of the first. The isolate was identified in three of five replicates as the first rank with a SMI range of 0.76-0.82. The other two replicates had as the first rank *Salmonella enteritidis*, followed closely (SMI difference <0.1) by *E. coli*. Similarly, in the three SLI-correct replicates, the first rank was followed by *S. enteritidis* and the SMI difference was <0.1 unit. This closeness among these two species is also observed in 16S rRNA studies (Scheutz and Strockbine, 2005). Although there was a high standard deviation in mean 17:0 cyclopropane and SF 3 contents (Table 2.6.), both fatty acid ranges did not seem to impact the identification of this strain. The RTSB 50 library consists of six separate subgroups (A to F) for *E. coli* separated by quantitative differences within major fatty acids and the presence of some minor (<1.0%) fatty acids. Brown and Leff (1996) found that Sherlock MIS had a good ability to identify aquatic non-pathogenic strains of *E. coli*. Odumeru et al. (1999) reported that MIS could only identify 53% of forty verotoxigenic

E. coli strains. Steele et al. (1997) stated that fatty acid profiles have a limited use in clearly distinguishing between the subgroups of verotoxigenic *E. coli* and *S. enteritidis*. Based on the fatty acid content, *E. coli* ATCC 11303 was identified as subgroup A for all replicates. Although there was wide variation for individual fatty acid quantities within replicates, the identification of this particular strain was achieved. Although Sherlock MIS had the ability and reproducibility to identify *E. coli*, further testing would be required to confirm separation of *E. coli* from *S. enteritidis*.

2.4.3.4. Hafnia alvei 12E1 and 2F1

Sakazaki (1984b) classified the genus *Hafnia* under family Enterobacteriaceae and defined it as the genus possessing gram-negative, catalase-positive, oxidase-negative, motile, straight rods with a G+C of 48-49 mol%. There has been only one species, *H. alvei* (Janada and Abbott, 2006), described in this genus with two distinct hybridization groups (Janada et al., 2005). This organism has been isolated from clinical specimens, mammals, birds, fish and a variety of food sources (Rodriguez et al., 1999).

2.4.3.4.1. Fatty acid profile

The *H. alvei* strains isolated from surimi (2F1) and salmon pate (12E1) showed almost similar major fatty acid percentages (Table 2.6) except for a slight higher percentage of 17:0 cyclopropane fatty acid and SF 3 in *H. alvei* 2F1 (22% and 11%) compared to *H. alvei* 12E1 (18% and 15%). The major fatty acids of *H. alvei* are 16:0, 17:0 cyclopropane, SF 3 followed by 14:0, 18:1 ω7c and SF 2. Even the cumulative fatty acids are very similar except for a small difference in unsaturated fatty acids and cyclopropane which is due to the differences in 18:1 ω7c and 17:0 cyclopropane content. The 16:0, SF 3, 18:1 ω7c content in the present study is similar to the limited information given by Yrjala et al. (1998) on *H. alvei* ATCC 29927. Among the replicates of *H. alvei* 12E1, the 17:0 cyclopropane fatty acid content ranged from 11% to 23% with a concomitant change in SF 3 (i.e. 16:1 ω6c/ω7c) from 23% to 10%. The 16:0 content remained fairly constant

around 32-33% in all the replicates. Hence it seems logical that 16:1 may be formed through the *de novo* synthesis and that 17:0 cyclopropane is derived from 16:1. Similar observation was made in *H. alvei* 2F1 strain. The variability in the fatty acids represents the biological variation in the phenotypic expression of fatty acids by these strains and that this species might exhibit less reproducible and more-varied fatty acids even under standardized conditions (Steele et al., 1997).

2.4.3.4.2. Identification based on fatty acids

Strains 2F1 and 12E1 were preliminarily identified as *H. alvei* using API 20E with excellent identification. Among the five replicates of *H. alvei* 12E1, two replicates were identified as the first match with SMI of 0.842 and 0.838 (Table 2.2.); in another replicate the identification was a second choice in the possible identification list (SMI 0.698) which was < 0.1 SMI distance from the first rank. In the replicates identified as *Rahnella aquatilis* (SMI 0.693) as the first rank, '*Hafnia alvei*' was the fourth rank (SMI 0.521) which might be due to the slightly higher SF 2. The last replicate where only 93.23% peak percent named, the first rank was *R. aquatilis* (SMI 0.519) while *H. alvei* was the seventh rank (SMI 0.343) which can be possibly explained by the higher content of an unknown fatty acid at equivalent chain length (ECL) 14.502 and SF 2. Although, the content of 17:0 cyclopropane and SF 3 had a wide variation among the replicates and the mean of fatty acids for the *H. alvei* library entry, these fell within the percentage range of those fatty acid entries. Hence, the lower SMI would result from a deviation in fatty acid percentages (Figure 2.5.).

The isolate *H. alvei* 2F1 was identified correctly to the species level as the first rank in two replicates. For the other two replicates, the first rank was *R. aquatilis* (SMI 0.852 and 0.602) with *H. alvei* (SMI 0.654 and 0.388) had the peak percent named 94.3% and 93%, respectively. Similar to strain 12E1, there was a variation in 17:0 cyclopropane and SF 3 among the replicates but this variation fell within the respective fatty acid percentage range of the library entry. The variation in the identification in the last three replicates can be explained by the comparison chart (Figure 2.6.).

Albert et al. (1991) used API 20-E for the identification of *H. alvei* from diarrheal stool samples in Bangladesh but later Janada et al. (1999) found that these strains were related to genus *Escherichia* rather than *Hafnia* and stressed the importance of using multiple techniques for bacterial identification.

Based on the observations in fatty acids content and corresponding identifications it can be concluded that MIS has the ability to identify the *H. alvei* strains but do not exhibit reproducibility in strain identifications.

2.4.3.5. Serratia marcescens ATCC 13880 and *S. fonticola* 2D3

Facultatively anaerobic gram-negative straight motile rods which form opaque, somewhat iridescent and white, pink, or red colonies on nutrient agar have been classified under family Enterobacteriaceae as genus *Serratia* (Grimont and Grimont, 2005a). These bacteria are found in the environment and are opportunistic human pathogens (Grimont and Grimont, 2005a) and plant pathogens (Rascoe et al., 2003).

2.4.3.5.1. Fatty acid profile

Serratia fonticola 2D3 isolated from surimi and *S. marcescens* ATCC 13880 showed similar content for major fatty acids of 16:0, SF 2 and SF 3 (Table 2.6.). Differences between *Serratia* spp. showed *S. marcescens* had higher 18:1 ω7c (17%) and only traces (<1%) of 15:0 and 17:0 cyclopropane. The fatty acid 14:0 2-OH was absent and 19:0 cyclopropane ω8c was <1% for *S. fonticola* 2D3. For all replicates of *S. marcescens*, the 16:0 content remained fairly constant (about 28-29%) while the 17:0 cyclopropane varied (5-11%) with a concomitant change in SF 3. In contrast, although the 16:0 content of *S. fonticola* remained constant, the changes in 17:0 cyclopropane content was reflected in the changes in SF 3 and 15:0 percentages.

2.4.3.5.2. Identification based on fatty acid profile

Serratia marcescens ATCC 13880 was identified correctly in three of five replicates with one limited to genus level and the other misidentified (Table 2.3.). Of three correctly identified replicates, *S. marcescens* was the first rank once while *Salmonella choleraesuis* subsp. *houtenae* and *Klebsiella pneumoniae* was the first rank for the other two replicates separated by >0.1 SMI from *S. marcescens*. An inspection of the fatty acid profiles and the comparison chart (Figure 2.7.) showed that for ATCC 13880 to be identified as the first rank, 19:0 iso, 16:0 anteiso and SF 6 which were relatively minor fatty acids should be present. Of these, one or two fatty acids were absent in the other two replicates (GLI and mismatch). Analysis of chromatograms of the later replicates showed these fatty acid peaks were present but not integrated by the software. In addition, one of the later two replicates had a higher 18:1 ω7c and SF 3 content than the range used by Sherlock MIS (Figure 2.7.). For the other correctly identified replicate, all fatty acids were in the range of the library entry but lacked 16:0 anteiso and 19:0 and had only trace amounts of 11:0 3-OH, 13:0, 13:0 2-OH, 15:0 3-OH and 18:1 ω5c. One replicate to GLI (SMI of 0.53) listed as *S. rubidae* was due to the lower content of 16:0 (27%) and slightly higher percentage of 15:0 (1.25%) than the fatty acid range in the *S. marcescens* RTSB50 library entry. In addition, the presence of 13:0, 13:0 2-OH and 11-methyl 18:1 ω7c in minor proportions may have interfered in the identification of this strain. The replicate which was not identified can be due to the absence of 19:0 iso and C16:0 anteiso; a lower percentage of 16:0 (27%) and higher percentage of SF 2 (11%) as compared to the comparison chart of the library entry of *S. marcescens* and the presence of 13:0. For all replicates, the listing provided by the Sherlock MIS consisted of bacteria belonging to Enterobacteriaceae. Tas et al. (1988) stated although *S. marcescens* is a potentially interfering strain in the taxonomic identification of *Salmonella* spp., the fatty acid data can be effectively used for the separation of these two genera. Langsrud et al. (2003) found fourteen stains isolated from footbaths in the Norwegian dairy industry were identified initially as *Cedecea* spp. (SMI >0.8) by Sherlock MIS were confirmed as *S. marcescens* by Rapid ID 32E API analysis and 16S-rDNA sequencing. Similarly, *S.*

marcescens ATCC 13880 showed some matches to *C. davisae* (SMI of 0.48-0.6) and *C. neteri* (SMI of 0.59-0.65).

Serratia fonticola 2D3 isolated from surimi, previously identified using API RapidE (7077270 = excellent identification), showed ambiguous results (average SMI <0.5) in five replicates (Table 2.2). In the first replicate the peak percent named was 93%. This replicate was identified as *S. typhimurium* GC subgroup B (poor SMI of 0.16) as the first rank followed by *S. fonticola* (poor SMI of 0.14). The second replicate was identified as *S. fonticola* (fair SMI of 0.36) as the first rank followed by *S. typhimurium* GC subgroup B (poor SMI of 0.23). The third replicate was identified as *S. typhimurium* GC subgroup B (SMI = 0.36) followed by *E. intermedius* (fair SMI of 0.31) and *S. fonticola* (poor SMI of 0.3). For the remaining two replicates, *Yersinia aldovae* (SMI of 0.7) and *S. fonticola* (SMI of 0.6) were the first two ranks. The main reason for the lower SMI in three replicates was due to 15:0, 16:0 and SF 3 were outside the range for *S. fonticola* (Figure 2.8.).

Although the Sherlock MIS has the library entry for *S. marcescens* and *S. fonticola*, the identification of these isolates were questionable.

2.4.4. Gram-negative, oxidase-positive bacteria

2.4.4.1. Aeromonas hydrophila ATCC 35654

Baumann and Shubert (1984) classified genus *Aeromonas* under family Vibrionaceae and confined the genus to gram-negative, catalase-and oxidase-positive, straight rods with rounded shapes and coccus generally motile by single flagella. The genus was divided into two major groups, one consisting of psychrophilic and non-motile *Aeromonas salmonicida* (three subgroups) and the other containing mesophilic and motile *A. hydrophila*, *A. sobria* and *A. caviae* (Popoff, 1984). Colwell et al. (1986) reported enough genetic differences exist between *Aeromonas* and other members of Vibrionaceae and

Enterobacteriaceae to recommend formation of the Family Aeromonadaceae. Fourteen genomes have been described in this family (Abbott et al., 2003). *Aeromonas* isolated from the aquatic habitat are known as disease-causing agents in fish and humans (Martinez-Murcia et al., 2005). Aeromonads are found in skin, gills and intestines of aquacultured striped bass (Nedoluha and Westhoff, 1995), vegetables, red meat, poultry, meat products, fish and shrimp in retail markets (Neyts et al., 2000), raw and cold-smoked salmon and trout (Gonzalez-Rodriguez et al., 2002), raw and processed seafood products in the retail markets (Ullmann et al., 2005), and prepacked conger, swordfish, sole grouper and whiting (Herrera et al., 2006).

2.4.4.1.1. Fatty acid profile

Aeromonoas hydrophila ATCC 35654 had 34% of total saturated fatty acids and 57% of total unsaturated fatty acids (Table 2.7.) and lacked cyclopropane fatty acids. The prominent fatty acids in ATCC 35654 were 16:0 (17%), and SF 3 (36%) which were similar to *A. popoffii*, *A. hydrophila* homology group (HG) 1 and 3 and *A. bestiarum* (Huys et al., 1997). For ATCC 35654, 12:0 was similar to *A. hydrophila* HG1 while higher than HG3 and 18:1 ω7c was higher than HG1 and HG3 (Huys et al., 1997).

Based on fatty acid biosynthesis, the FAS II pathway for 16:0 was converted to 16:1 ω7c and elongated to 18:1 ω7c or *de novo* synthesis of 16:0 and 16:1 and the later elongated to a 18:1 fatty acid.

2.4.4.1.2. Identification based on fatty acid profile

Sherlock MIS identified all replicates of *A. hydrophila* ATCC 35654 correctly with a SMI of 0.89 (Table 2.3.). The first rank was *A. ichthiosmia* / *A. hydrophila* which were closely followed by other *Aeromonas* spp. in the RTSB50 library. All fatty acid quantities for the replicates had a standard deviation of <1 except for SF 2 and SF 3 (Table 2.7.). It can be stated that *A. hydrophila* was a repeatable identification. Kuhn et al. (1997) reported isolates from drinking water were identified as *A. hydrophila* by Sherlock MIS and confirmed using API 20NE. Hinton et al. (2004) used the Sherlock MIS to identify *A.*

sorbia, A. ichthiosmia, A. caviae, A. hydrophila, A. salmonicida subsp. *masoucida* and *A. shubertii* during spoilage bacteria of refrigerated poultry. Neyts et al. (2000) used the Sherlock MIS system solely for aeromonad identification in various foods.

2.4.4.2. Alteromonas sp. 8C2, *Pseudoalteromonas nigrifaciens* ATCC 19375 and *Shewanella putrefaciens* ATCC 8071 and ATCC 49138

The bacteria classified under genus *Alteromonas* are gram-negative, straight or curved motile rods with a G+C content of 38-50 mol% DNA (Baumann et al., 1984). Van Landshoot and DeLey (1983) demonstrated three distinct rRNA similarity groups within *Alteromonas* and proposed forming a new genus *Marinomonas*. MacDonell and Colwell (1985) proposed a new genus *Shewanella* based on 5S RNA sequence analysis and assigned two species, *A. putrefaciens* and *A. hanedai*. Many species have been added to this genus from various sources (Yoon et al., 2004; 2003b). The current taxonomic classification of *S. putrefaciens* has evolved in its identity initially in 1931 as *Achromobacter putrefaciens* to *Pseudomonas putrefaciens* to genus *Alteromonas* (Stenström and Molin, 1990). Gauthier et al. (1995) conducted rRNA studies on seventeen species of *Alteromonas* and proposed forming a new genus *Pseudoalteromonas* which included eleven species from the original *Alteromonas* and one from *Pseudomonas* and proposed *Pseudoalteromonas haloplanktis* ATCC 14393 as the type species. *Alteromonas* is restricted to *A. macleodii* (Gauthier et al., 1995), *A. marina* (Yoon et al., 2003b), *A. litorea* (Yoon et al., 2004), *A. stellipolaris* (Trappen et al., 2004) and *A. addita* (Ivanova et al., 2005). Ivanova et al. (2004) used polyphasic taxonomic studies to proposed the family Alteromonadaceae which includes *Alteromonas* and *Glacicola*; family Pseudoalteromonadaceae which included *Pseudoalteromonas* and *Algicola* while family Shewanellaceae contained a single genus.

2.4.4.2.1. Fatty acid profile

The fatty acid profiles of *Alteromonas* sp. 8C2, *P. nigrifaciens* ATCC 19375, and *S. putrefaciens* ATCC 8071 and 49138 are shown in Table 2.7. Strain 8C2 and both *S. putrefaciens* strains had very similar fatty acid profiles. The major fatty acid content of *Alteromonas* sp. 8C2 and the *S. putrefaciens* strains comprised 15:0, 16:0, 13:0 iso, 15:0 iso, 17:1 ω8c and SF 3 and resembled those for two *Shewanella* species reported by Khashe and Janada (1998). There were quantitative differences among the fatty acids with respect to higher 15:0 content (14-23%) while 16:0 content was within the range for *Alteromonas* sp. 8C2 but was higher for *S. putrefaciens* ATCC 8071 and ATCC 49138. Compared to these three strains, *P. nigrifaciens* had higher 12:0 3-OH, 15:1 ω8c, 16:0, and SF3 and higher degrees of saturation (34%) and unsaturation (49%) while a low degree of branched chain fatty acids (5%). These fatty acids can clearly distinguish *P. nigrifaciens* from *Alteromonas* sp. 8C2 and the *S. putrefaciens* ATCC strains. Yoon et al. (2004) observed *A. macleodii*, *A. marina* and *A. litorea* have high concentrations of 16:0 (20-24%) and 18:1 ω7c (10-12.5%) and a low concentration of 17:1 ω8c (3.6-5.6%), as compared with the four strains in this test group.

2.4.4.2.2. Identification based on fatty acid profile

Alteromonas sp. 8C2, identified only to the genus level using classical microbiological techniques, was isolated from flatfish in 1990 and exhibited proteolytic, lipolytic activity and was hydrogen sulfide-positive on peptone iron agar. This strain was identified as *S. putrefaciens/alga*, the only entry for the genus *Shewanella*, with an average SMI 0.69 (Table 2.7). Although the genus *Alteromonas* still exists, there is no separate entry for any bacterium belonging to *Alteromonas* in the RTSB50 library. Khashe and Janada (1998) have shown that quantitative differences exist between the major fatty acid contents of *S. alga* and *S. putrefaciens* with respect to 15:0 (26% 18%), 16:0 (7% vs 11%), 17:1 ω8c (15% vs 12%) and 12:0 (2% vs 3%). Even though the average fatty acid profile of *Alteromonas* sp. 8C2 falls within the range of *S. alga* and *S. putrefaciens*, *S.*

alga is mostly related to clinical samples while *S. putrefaciens* is associated with non-human sources (Khashe and Janada, 1998). Hence, in consideration of the previous biochemical tests performed and the taxonomic changes in the position of *Alteromonas*, the identification of *Alteromonas* sp. 8C2 was confirmed as *S. putrefaciens*.

Shewanella putrefaciens ATCC 8071 and ATCC 49318 were identified as *S. alga/putrefaciens* with SMI of 0.77 and 0.96, respectively (Table 2.3.). The SMI for ATCC 49318 was close to 1.00 which denotes that this strain probably was used to create the library entry. The slightly lower SMI for ATCC 8071 was due to the lower percentages of C15:0 iso (16% versus 24% for the RTSB50 library entry) and 17:1 ω8c (14% versus 20% for library entry) and a higher SF3 (19% versus 10% for library entry).

Pseudoalteromonas nigrifaciens ATCC 19375 was correctly identified to the species level with a SMI of 0.85 (Table 2.3.). There are six species in the RTSB50 library for *Pseudoalteromonas* separated by qualitative, quantitative and presence or absence of fatty acids.

Since all replicates of the four test strains were correctly identified, the ability of MIS to identify these bacteria was reproducible.

2.4.4.3. Flavobacterium sp. 5C6

Genus *Flavobacterium* are defined as gram-negative, catalase-, oxidase- and phosphatase-positive, aerobic, non-motile and non-gliding rods with a low G+C content of 31-42 mol% DNA (Holmes et al., 1984). Bernardet et al. (1996) emended *Flavobacterium* to consist of bacteria which can utilize a wide variety of polysaccharides except cellulose and can use peptone as a source of nitrogen and carbon. Vancanneyt et al. (1996a) introduced a new genus, *Myroides*, which contained gram- negative, catalase-, oxidase-, urease-, and gelatinase- positive aerobic rods lacking flagella and gliding movement. Based on polyphasic taxonomic studies, Vancanneyt et al. (1996a) included

F. odoratum renamed as *Myroides odoratus*. Recently, Yoon et al. (2006) introduced a new species *M. pelagicus* which was isolated from seawater.

2.4.4.3.1. Fatty acid content

The predominant fatty acid profile for *Flavobacterium* sp. 5C6 and the cumulative fatty acid data is shown in Table 2.7. Strain 5C6 had a high percentage of 15:0 iso (53%) with relatively smaller proportions of 17:0 iso 3-OH (11%) and SF 4 (17%). The fatty acid profile of *Flavobacterium* sp. 5C6 differed from Antarctic lake isolates *F. gillisiae* and *F. tegetinola* (McCammon and Bowman, 2000; McCammon et al., 1998). The cultivation, extraction and quantification techniques used by these researchers are different than the MIDI protocol used in the present study which challenges the ability to compare species within the same genus (Haack et al., 1994). Rasoamananjara et al. (1988) conducted principal component analysis of fatty acid data of various species of *Flavobacterium* which revealed six of eight strains of *F. odoratum* clustered together.

The fatty acid profiles of *M. odorous*, *M. odoratimimus* and *M. pelagicus* (Vancanneyt et al., 1996a; Yoon et al., 2006) can be used for comparison with the present study as these researchers had used the standard MIDI protocols with the exception of using an earlier version of Sherlock MIS software. Before comparing the fatty acid profiles for *Myroides* spp. with *Flavobacterium* sp. 5C6, the profiles for type stain *M. odoratus* ATCC 4651 were compared. There were differences in the fatty acid profiles for the same bacterium (LMG 1233 = JCM 7458 i.e. both these strains from different collection are the same) grown for 48 h and 24 h at two different laboratories. The fatty acids for 15:0 iso (46% vs 52%), 17:0 iso 3-OH (21% vs 11%) and 17:1 ω9c (14% vs 21%) were observed from the data of Yoon et al. (2006) and Vancanneyt et al. (1996a), respectively.

The average fatty acid profile of *Flavobacterium* 5C6 was very similar to *M. odoratus* ATCC 4651 (Vancanneyt et al., 1996a) except for higher 16:0 3-OH (4% compared to 1.2%) and SF 3 (2% compared to <1%) and lower 17:1 iso ω9c (17% compared to 21%). Strain 5C6 differed from *M. odoratimimus* (Vancanneyt et al. 1996a) in terms of trace

amounts of 13:0 iso compared to 7% and higher 15:0 iso (52% compared to 45%) and 17:1 iso ω9c (17% compared to 10%). Comparison with the fatty acid data of *M. odoratus, M. odoratimimus* and *M. pelagicus* (Yoon et al., 2006a) showed that the predominant fatty acid content of *Flavobacterium* sp. 5C6 fell within the range of the three bacteria.

2.4.4.3.2. Identification based on fatty acid profile

Gram-negative, oxidase-positive rods forming yellow to orange colonies isolated from Alaska pollock were presumptively designated as *Flavobacterium* sp. in 1990. Based on the cellular fatty acid profile, strain 5C6 was identified in the five replicates as *M. odoratus* (*F. odoratum*) with an average SMI 0.62 (Table 2.2.). The RTSB50 library has five entries in the genus *Flavobacterium*, with only one entry in genus *Myroides*. Strain 5C6 was not identified under this genus due to the presence or absence and quantitative differences within the fatty acid profile. The fatty acid profile comparison chart of *M. odoratus* showed that most of the fatty acids of strain 5C6 fell within the percentage range of the library entry.

Based on the average fatty acid profile of *Flavobacterium* sp. 5C6, the comparisons with literature and the identification given by the Sherlock MIS library, this strain belongs to genus *Myroides* and assigned possibly as a strain of *M. odoratus* or *M. odoratimimus*. Since the identifications of all replicates were constant, strain 5C6 results in laboratory reproducibility.

2.4.4.4. Moraxella sp. 5E6 and *Psychrobacter immobilis* ATCC 43116

The genus *Moraxella* was classified under family Neisseriaceae and consisted of two oxidase-positive and usually catalase-positive subgenus divided into *Moraxella* (rods) and *Branhamella* (cocci) producing non-pigmented colonies (Bovre, 1984). *Moraxella*-like strains initially categorized as *Achromobacter* isolated from fish, poultry, irradiated

food, seawater and other sources are gram-negative, non-motile psychrotrophs distinct from *Acinetobacter* and *Moraxella* (Juni and Heym, 1980). *Moraxella*-like strains isolated from fish and processed meat and poultry products are reclassified into a new genus *Psychrobacter* (Juni and Heym, 1986). Hudson et al. (1987) found that *P. immobilis* is found in foods and can be isolated from clinical specimens. The main difference between *Psychrobacter* and *Moraxella* is the optimum growth temperature of 20-25°C (Juni and Heym, 1986) versus 30-35°C (Bovre, 1984), respectively. In addition, qualitative and quantitative fatty acid differences can be used to distinguish between the clinical strains of *Acinetobacter*, *Moraxella* and *P. immobilis* (Moss et al., 1988). The main distinguishing factor between *P. immobilis* and *Moraxella* is the presence or absence of 17:0 iso fatty acid, respectively, and *Acinetobacter* can be distinguished from both by the presence of 12:0 2-OH (Moss et al., 1988). Rossau et al. (1991) proposed *Psychrobacter*, *Moraxella* and *Acinetobacter* should be included in a new family *Moraxellaceae* based on DNA-RNA hybridization. Bowman et al. (1996) confirmed that *M. phenylpyruvicus* is closely related to *P. immobilis* and renamed the former as *P. phenylpyruvicus*. A number of species have been collected from the marine psychrotrophic and psychrophilic environment, such as *P. urativorans* and *P. frigidicola* (Bowman et al., 1996), *P. glacincola* (Bowman et al., 1997), *P. pacificensis* (Maruyama et al., 2000), *P. submarinus* and *P. marincola* (Romanenko et al., 2002), *P. luti* and *P. fozii* (Bozal et al., 2003) *P. okhotskensis* (Yumoto et al., 2003), *P. celer* (Yoon et al., 2005a), *P. aquimaris* and *P. namhaensis* (Yoon et al., 2005b). From seafood, *P. proteolyticus* was isolated from krill (Denner et al., 2001) and *P. jeotgali* (Yoon et al., 2003a) and *P. alimentarius* (Yoon et al., 2005c) were discovered in fermented seafood. Also, *P. faecalis* was isolated from bioaerosol originating from pigeon feces (Kampfer et al., 2002) and *P. pulmonis* from lungs of lambs (Vela et al., 2003), and *P. cryohalolentis* and *P. arcticus* were isolated from the Siberian permafrost (Bakermans et al., 2006). This wide distribution of *Psychrobacter* in the marine environment is due to their ability to grow at low temperatures, metabolize a variety of substrates and are halotolerant (Yumoto et al., 2003).

2.4.4.4.1. Fatty acid profile

Moraxella sp. 5E6, isolated from pollock and *P. immobilis* ATCC 43116 were compared for fatty acid composition (Table 2.7). Cyclopropane fatty acids were completely absent while only one branched-chain hydroxyl fatty acid (13:0 iso 3-OH) was present in trace amounts. Although fatty acid profiles were similar, strain 5E6 contained 12:0 but was absent in *P. immobilis*. *Moraxella* sp. 5E6 when compared to *P. immobilis* had higher percentages of SF 3 (16% vs 4%) and 17:0 iso (4% vs 2%) and lower percentage of 18:1 ω9c (48% vs 63%). In addition, both strains had trace quantities of highly unsaturated fatty acids (3-4 double bonds) which were absent for all other bacteria in the present study. The predominance of 18:1 ω9c, 17:1 ω8c and SF 3 fits *Moraxella* 5E6 into the genus *Psychrobacter* (Bozal et al., 2003). Comparison of the present data for strains 5E6 and 43116 with the average (n=14) fatty acid profiles of *P. immobilis* strains reported by Moss et al. (1988) showed differences in the presence and amounts of 18:3 ω6c, 18:2, 19:1, 16:1 ω7c. Although various researchers have used the MIDI protocol for extraction of fatty acids, the incubation time and temperatures vary in addition to species and may explain the differences in FAME data. For example, Moss et al. (1988) cultured their isolates at 35°C for 24 h on brain heart infusion agar containing 5% rabbit blood while in the present study, TSBA with no blood supplement was used and plates were incubated at 28°C for 24 h.

2.4.4.4.2. Identification based on fatty acid profile

All replicates of *Moraxella* sp. 5E6 and of *P. immobilis* ATCC 43116 were identified as *P. immobilis* (Table 2.2. and Table 2.3., respectively). Library entries for the genus *Moraxella* in the RTSB50 library include, *M. catarrhalis* (*Branhamella catarrhalis*), *M. nonliquefaciens, M. osloensis*, and *M. ovis*. These entries had higher 16:0 (7%) and SF 3 (21%) (for *M. catarrhalis*); presence and high percentage of 14:0 (9%), 16:0 (30%), 17:0 cyclopropane (12%), 19:0 cyclopropane (<1%), SF 2 (8%) (for *M. nonliquefaciens*); higher 10:0 (4%) and 18:1 ω9c (74%) and absence of 17:1 ω8c and any branched chain

fatty acid (for *M. osloensis*); and higher 12:0 3-OH (6%), 16:0 N alcohol (3%), 16:0 (12%) and 18:3 ω6c (5%) (for *M. ovis*). Library entries for *Psychrobacter* include *P. phenylpyruvicus* which differed due to higher percentage of 11:0 (4%), 11:0 iso (2%), 12:0 3-OH (7%), 16:0 (11%), 18:0 (5%), 20:4 (3%) and SF 6 (22%) and lower amounts of 17:1 ω8c (<1%) and 18:1 ω9c (21%). The fatty acid profiles of both strains matched well with the library entry of *P. immobilis* hence were identified as *P. immobilis* rather than *P. phenylpyruvicus*. The average SMI of 0.9 for *P. immobilis* ATCC 43116 was very high indicated that its fatty acid profile was closer to the average fatty acid profile of the library entry (Figure 2.9.) and that this strain might have been used for developing the library. The lower SMI of 0.53 for *Moraxella* sp. 5E6 was attributed to the higher percentages of 16:0 and SF 3 which fell near the upper limit of the given range for these fatty acids for library comparison. Additionally, low 18:1 ω9c content, absence of SF 6 (18:0 anteiso/18:2 ω6,9c) and presence of low percentage of SF 7 might have contributed to lower SMI. Similarly, Pacova et al. (2001) reported ten *P. immobilis* strains isolated from cheese, poultry carcass, irradiated sausage, and salted fish had SMI ranging 0.39 to 0.61 with only one isolate from cheese being identified with a SMI of 0.78. Pacova et al. (2001) had used strains isolated from raw and spoiled fish (from cod and another fish species not reported) but did not report the identifications from MIS. The isolates from salted fish had SMI of 0.54-0.57 while one isolate did not show a match with the library.

Psychrobacter immobilis has been isolated from lamb carcass (Prieto et al., 1992) and chilled storage of aquacultured rainbow trout, wild brown trout and wild pike (Gonzalez et al., 2000). Also, *P. phenylpyruvicus* and *P. urativorans* and *Psychrobacter* spp. which resembled *P. urativorans* were identified (Gonzalez et al., 2000). In consideration of the fatty acid data, the identifications given by Sherlock MIS, and comparison with the literature, *Moraxella* sp. 5E6 should be renamed as *P. immobilis*. Sherlock MIS was able to correctly and reproducibly identify *P. immobilis* ATCC 43116 which is the type strain.

2.4.4.5. Providencia alcalifaciens 7F2

This bacterial strain was isolated from spoiled herring and identified using API 20E as *Providencia alcalifaciens* with an excellent identification (API 20E code 0264000, Himelbloom, personal comm.). The same strain was tested on the Sherlock MIS system for its fatty acid profile and confirm its identity.

2.4.4.5.1 Fatty acid profile

The fatty acid profile comprised primarily SF 3 (37%), 16:0 (29%), 18:1 ω7c (12%) and 14:0 (6%). The iso, anteiso and cyclopropane fatty acids were either absent or present in trace quantities (Table 2.7.). MacTiger and O'Leary (1973) reported *P. alcalifaciens* in its stationary phase is comprised of 16:1 (31%), 16:0 (30%), 19:0 cyclopropane (16%), 14:0 (14%), 18:1 (4%), 15:0 cyclopropane (2%) and 14:1 (2%).

2.4.4.5.2 Identification based on fatty acid profile

All five replicates were identified as *Erwinia chrysanthemi*-biotype III as the first rank with an average SMI of 0.75. The possible identification list of three replicates had a match as *Providencia rustigianii* which was <0.1 SMI unit different from the first rank; in one replicate, the second rank was <0.1 SMI of *E. chrysanthemi*. Since strain 7F2 was identified only to the genus level, the average fatty acid profile was superimposed on the library comparison charts of *P. alcalifaciens* (Figure 2.10) and *P. rustigianii* (Figure 2.11) to illustrate the closeness of the observed fatty acid profiles and the library entries for these bacteria. Most of the fatty acid quantities were within or closer to the range of fatty acids for *P. rustigianii* library entry than those for *P. alcalifaciens*.

There is no DNA relatedness between the *Providencia* and *Erwinia* (Figure 5.2. in Brenner, 1984) and there is no taxonomic crossover of *P. alcalifaciens* and *E. chrysanthemi* (O'Hara et al., 2000). The possibility of misidentification due to change in taxonomy is ruled out. Although the API 20E results gave an "excellent" level of identification of the test strain as *P. alcalifaciens*, the Sherlock MIS system identified the

strain as *E. chrysanthemi*. Hence ambiguity exists about the exact taxonomic position of strain 7F2 and further analysis should be conducted to ascertain the identity of this bacterium.

2.4.4.6. Morganella morganii 2E5

Classified under Gammaproteobacteria in family Enterobacteriaceae, the genus *Morganella* consists of gram-negative, catalase-positive, oxidase-negative, motile straight rods and the type strain *M. morganii* is divided into biogroups A to G (Janda and Abbott, 2005). Histamine is produced by this species in seafoods and causes a chemical intoxication leading to illness (Kim et al., 2002; O'Hara et al., 2000). A novel, psychrotrophic, histamine-producing species *M. psychrotolerans* was isolated from seafoods (Emborg et al., 2006).

2.4.4.6.1. Fatty acid profile

The major fatty acids in *M. morganii* 2E5 were 16:0 (32%), 17:0 cyclopropane (18%), SF 2 (10%), SF 3 (9%) and 18:1 ω7c (9%) (Table 2.7.). Vasyurenko and Chernyavskaya (1990) reported the major fatty acids in *M. morganii* are 16:0, 18:1 and anteiso 16:0 which differs from the composition observed for strain 2E5. The major fatty acid profiles of *M. psychrotolerans*, *M. morganii* and its two subspecies reported by Emborg et al. (2006) are similar with respect to 16:0 in the present study while 16:1 ω7c, 17:0 cyclopropane and 18:1 ω7c fell within the range of fatty acids of the 2 species.

2.4.4.6.2. Identification based on fatty acid profile

Morganella morganii 2E5 was isolated from surimi and identified previously using API 20E (code number 0174000 = excellent identification; Himelbloom, personal comm.). Among the five replicates, three were identified correctly as *M. morganii* as the first rank (SMI range of 0.49-0.71). The comparison charts of these replicates showed the SMI increased when 18:1 ω7c and SF 2 were closer to the average percentage of these fatty

acids in the library entry (Figure 2.12.). The other two replicates were identified as *Salmonella typhimurium*-GC subgroup B (SMI of 0.68) and *Ewingella americana* (SMI of 0.73) and separated from *M. morganii* by 0.23-0.32 SMI units (Table 2.2.). It can be concluded that Sherlock MIS has the capability of correctly identifying *M. morganii* 2E5.

2.4.4.7. Pseudomonas fluorescens and P. putida strains

Bacteria belonging to the genus *Pseudomonas* are aerobic, gram-negative, catalase- and oxidase-positive motile rods possessing one or several flagella (Palleroni, 1984). Pseudomonads identified using RNA-rRNA/DNA homology were divided into five groups (Palleroni et al., 1973). With the changes in taxonomy, only RNA group I comprises of species of *Pseudomonas* (Anzai et al., 2000). The RNA group II organisms were transferred to *Burkholderia* (Yabuuchi et al., 1992), *Ralstonia* (Yabuuchi et al., 1995) *Comamonas* (Tamaoka et al., 1987), *Acidovorax* (Willems et al., 1990) and *Hydrogenophaga* (Willems et al., 1989), RNA group IV were transferred to *Brevundimonas* (Segers et al., 1994) while *P. maltophila* (RNA group V) was renamed *Stenotrophomonas maltophila* (Palleroni and Bradbury, 1993).

Non-phytopathogenic *Pseudomonas* (267 strains) were divided by Stanier et al. (1966) into *P. fluorescens* (biotypes A to G) and *P. putida* (biotypes A and B). Biotypes A, B, C, F and G are referred to as biovar I, II, III, IV and V, respectively, while biotypes D and E are *P. chlororaphis* and *P. aureofaciens* (Palleroni, 1984). Based on the substrate utilization patterns, Barrette et al. (1986) proposed the creation of *P. putida* biovar/biotype C for food spoilage strains grouped under *P. fluorescens* biovar V. This newly formed biovar has not been mentioned by Bossis et al. (2000) in the taxonomic review of *P. fluorescens* and *P. putida*.

Stanier et al. (1966) experimented with identifications of isolates mainly from the aquatic environment and Shaw and Latty (1982) conducted a numerical taxonomic study of 160 different characters for pseudomonads isolated from aerobically spoiled beef and pork.

The meat strains had a different carbon substrate utilization patterns than those of Stanier et al. (1966). In contrast, Molin and Ternström (1986) found 93% of fluorescent strains isolated from spoiled meat, 33% from water and 60% from soil were similar to the biovars described by Stanier et al. (1966). Gennari and Dragotto (1992) were unable to identify 11% of the 445 fluorescent strains isolated from the microflora of fresh and spoiled meat and fish, raw milk, cheese, soil and water using twelve different carbon sources and other specific tests. Ternström et al. (1993) studied raw and spoiled bovine milk and Arnaut-Rollier et al. (1999) studied poultry meat and found clusters of *Pseudomonas* strains separated from known biovars but closely related to *P. fluorescens* biovars A and C. Tryfinopoulou et al. (2002) demonstrated the clusters of *P. fluorescens*, *P. putida* and *P. fragi* are indistinguishable based on physiological characteristics and whole-cell protein profiles.

Genus *Pseudomonas* is very diverse and can utilize a wide range of substrates (Palleroni and Bradbury, 1993). This diversity can be attributed to exposure of spatially and temporally complex environments (Spiers et al., 2000). Fatty acid content (Moss and Dees, 1976; Oyaizu and Komagata, 1983), DNA sequencing (Hsueh et al., 1998), whole cell protein analysis (Tryfinopoulou et al., 2002), DNA-DNA hybridization (Humphry et al., 2003), and 16S rRNA sequencing (Garcia-Lopez et al., 2004) have been used to unravel the taxonomy of *Pseudomonas*.

2.4.4.7.1. Fatty acid profile

Ten fluorescent *Pseudomonas* comprising *P. fluorescens* and *P. putida* strains (Table 2.1.) were compared for whole cell fatty acid compositions (Table 2.8.). All strains had similar fatty acid profiles with quantitative differences except for *P. putida* 7E4. The seven *P. fluorescens* strains had a wide variation in 17:0 cyclopropane (trace to 9%) while 18:1 ω7c varied from 9-20%. In the *P. putida* strains, the presence of 17:1 ω7c (18%) and absence of 18:1ω7c were the major differences between *P. putida* 7E4 and two other strains. Although the *P. putida* strains had higher average 17:0 cyclopropane content and lower SF 3 than *P. fluorescens* strains, there was no difference between the

fatty acid profiles of both species (Table 2.8.). The *P. fluorescens* FITC strains had higher 17:0 cyclopropane content compared to ATCC 13525 and ATCC 17574.

All strains had saturated, saturated hydroxyl, cyclopropane and unsaturated fatty acids but the iso and anteiso fatty acids were absent. The existence and quantitative difference in these fatty acids can form a distinguishing feature between different *Pseudomonas* strains (Moss and Dees, 1976). The presence of branched chain hydroxyl fatty acids, i.e., 11:0 iso 2-OH, 3-OH, 12:0 iso 3-OH and 13:0 iso 3-OH is a distinguishing characteristic of *P. maltophila* (Moss et al., 1973). High amounts of 18:1 (32.6%) distinguish *P. aeruginosa* from *P. fluorescens* and *P. putida* (Vancanneyt, et al. 1996b). Fatty acid content of *P. putida* ATCC 12633 in the present study differed quantitatively from the fatty acid analysis by Moss et al. (1972) with respect to the presence of 10:0 3-OH, 14:0, 16:1, 18:0 and 17:0 cyclopropane. Oyaizu and Komagata (1983) examined *P. putida* NCIMB 8859 and found 18:0, 19:0 and 19:0 cyclopropane fatty acids which were absent in the present study. These differences in the fatty acid content, between these studies and the present study, may be due to the differences in the source of the isolates, culture conditions and fatty acid extraction protocol. Vancanneyt et al. (1996b) used the MIDI protocol for the Sherlock MIS system to determine fatty acid content of *P. fluorescens* and *P. putida* strains. Although different proportions can be observed, the quantitative differences among fatty acids can be strain specific.

Oyaizu and Komagata (1983) proposed the importance of 3-OH fatty acids in grouping phytopathogenic and non-pathogenic *Pseudomonas* strains. Nine groups can be based on the combination of two or more and presence or absence of 8:0 3-OH, 10:0 3-OH, 12:0 3-OH, 14:0 3-OH, 14:1 3-OH, 16:0 3-OH, 11:0 iso 3-OH and 13:0 iso 3-OH (Oyaizu and Komagata, 1983). Group 1 comprises only those pseudomonads containing 10:0 3-OH and 12:0 3-OH and consisted of *P. fluorescens* and *P. putida*, fluorescent non-pathogenic strains, fluorescent phytopathogenic *P.* syringae, some non-pigment producing strains and several water-insoluble yellow pigment producing species. In the present study, the ten fluorescent *Pseudomonas* contained 10:0 3-OH and 12:0 3-OH and can be considered

as fatty acid group 1. Although 12:0 2-OH was observed in all strains (Table 2.8.), this fatty acid was not used by Oyaizu and Komagata (1983) for grouping fluorescent *Pseudomonas*.

Vancanneyt et al. (1996b) proposed seven groups of *Pseudomonas* based on whole cell and phospholipid fatty acid contents. The presence of 10:0 3-OH and 12:0 3-OH and absence of 14:0 3-OH are the diagnostic markers for rRNA group 1 proposed by Palleroni et al. (1973). The pseudomonads in the present study can be categorized as rRNA group 1.

2.4.4.7.2. Identification based on fatty acid profile

The identifications of the six FITC and four ATCC strains of *Pseudomonas* are given in Table 2.2. and Table 2.3., respectively. The superimposed comparison charts of the replicates which were not correctly identified (i.e. either SLI, mismatch or no ID) of *P. fluorescens* strains 11A1, 11B3, 12A2, and ATCC 31419 are given in Figures 2.13., 2.14., 2.15. and 2.16. respectively.

Pseudomonas fluorescens ATCC 31419 was identified only to the genus level in two replicates and species level in three replicates. All five replications had *P. putida* as the first rank in the identification list. The fatty acid profiles were superimposed on all entries in the RTSB50 library (*P. fluorescens* biotype A, B, C, F and G) to find out the possible reasons of the misidentifications. One replicate had a higher percentage (8%) of C17:0 cyclopropane and lower percentage (27%) of SF 3 than those in the library. A second replicate showed higher 10:0 3-OH, 12:0 2-OH, 3OH and 18:1 ω7c compared with the library entries and the three correctly identified replicates. Strain 11A1 was identified as *P. putida* as the first rank while *P. fluorescens* was in the possible identification list separated by >0.1 SMI unit in four of five replicates. The reason for one of the replicates not being identified as *P. fluorescens* was due to higher 17:0 cyclopropane (11%) and lower SF 3 (27%) contents (Figure 2.11.). Similarly the variation in the identification of

the replicates of *P. fluorescens* 11B3 and 12A2 can be explained through the comparison charts (Figures 2.12. and 2.13.).

The four FITC strains presumptively designated as *P. fluorescens* were identified correctly irrespective of the ranking in the possible identification list. Most of these correctly identified replicates showed the first rank as *P. putida*. This misidentification at the first rank and the identification of the isolates as *P. fluorescens* can be attributed to the closeness in the fatty acid profiles of these species to form a tight single cluster (Moss and Dees, 1976; Oyaizu and Komagata, 1983; Osterhout et al., 1991; Vancanneyt et al., 1996b). The variation in identification between the replicates can be due to the harvesting of cells from the stationary phase along with the log phase of the culture and can also be attributed to the inability of the system to distinguish between closely related species (Osterhout et al., 1991). Conversely, Hsueh et al. (1998) reported the cellular fatty acid method is effective to distinguish *P. fluorescens* and *P. putida* strain isolated from clinical specimen and these isolates had fatty acid profiles similar (SMI of 0.6-0.8) to *P. fluorescens* ATCC 13525 and *P. putida* ATCC 12633. The *Pseudomonas* strains used in the present study were isolated from the psychrotrophic environment and may change in fatty acid profile when these bacteria are grown at 28°C. Psychrotrophic *Pseudomonas* changes in the permeability of the membrane due to temperature can help in substrate uptake (Zachariah and Liston, 1973). This change in permeability is achieved by altering the fatty acid composition of the membrane (Morita, 1975). Gill (1975) observed that under carbon and nitrogen limited growth conditions, *P. fluorescens* increased 16:0 and 17:0 cyclopropane and decreased 16:1 and 18:1 fatty acids as the temperature was raised from 3°C to 30°C. Conversely, Herbert (1981) observed only one of three strains of psychrotrophic pseudomonads showed a 5% increase in 16:0 with a concomitant increase in 14:0 when the temperature was raised from 0°C to 20°C. The response of the psychrotrophs from the same genus is variable and strain dependent. This may explain the deviation of the fatty acid profile of the bacteria in the current study from the library to result in misidentification at the first match and lower similarity than clinical isolates of *Pseudomonas* (Hsueh et al., 1998).

Based on the literature, RTSB50 library entries and fatty acid profiles obtained, the identity of the *P. fluorescens* FITC strains belong in the *P. fluorescens/putida* complex (Osterhout et al., 1991). It is noted that the reproducibility of these bacteria is strain dependent.

2.5. Conclusion

A major aspect of using the MIDI rapid bacteria identification system is to reduce laboratory time which would be significantly longer for classical techniques. The fatty acid profile of organisms can help in creating and expanding the database of fatty acids of bacteria associated with seafoods. Fatty acid analysis can be used as a preliminary screening technique to identify the bacteria then other molecular techniques can be used to ascertain the exact taxonomic position of the bacterial isolate.

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Table 2.1. General characteristics of bacterial isolates used from the FITC Culture Collection (FITCCC) or obtained from the American Type Culture Collection (ATCC)

Gram-Positive Bacteria	Source or Isolated from	Colonies	Cells	Motility	Catalase	Oxidase
Arthrobacter sp. 5B6	Pacific pollock	Yellowish	R	−	+	+
Bacillus subtilis 5D1	Kodiak College Collection	Beige	R	+	+	+
Micrococcus varians 7F1	Smoked salmon	Yellow	C	−	+	−
Staphylococcus epidermidis	ATCC 14990	White	C	−	+	−
Staphylococcus xylosus 13A4	Fish food	White	C	−	+	−
Streptococcus faecium 6A4	"Stabisil" (commercial starter triculture)	White	C	−	−	−
Gram-Negative Bacteria						
Aeromonas hydrophila	ATCC 35654	Yellowish	R	+	+	+
Alteromonas sp. 8C2	Alaska flatfish	Yellowish	R	+	+	+
Citrobacter freundii 4E5	Alaska sole	Beige	R	+	+	−
Enterobacter cloacae 12D5	Dill weed	Beige	R	+	+	−
Escherichia coli	ATCC 11303	Beige	R	+	+	−
Flavobacterium sp. 5C6	Pacific pollock	Orange	R	−	+	+
Hafnia alvei 2F1	Pollock surimi	Beige	R	+	+	−
Hafnia alvei 12E1	Salmon paté	Beige	R	+	+	−
Moraxella sp. 5E6	Pacific pollock	Yellowish	C-B	−	+	+
Morganella morganii 2E5	Pollock surimi	Yellowish	R	+	+	+
Providencia alcalifaciens 7F2	Spoiled Pacific herring	Yellowish	R	+	+	+
Pseudoalteromonas nigrifaciens	ATCC 19375	White	R	+	+	+
Pseudomonas fluorescens	ATCC 17574	Yellowish	R	+	+	+

Table 2.1. contd...

Gram-Negative Bacteria	Source or Isolated from	Colonies	Cells	Motility	Catalase	Oxidase
Pseudomonas fluorescens	ATCC 13525	Yellowish	R	+	+	+
Pseudomonas fluorescens	ATCC 31419	Yellowish	R	+	+	+
Pseudomonas fluorescens 2A1	Pollock surimi	Yellowish	R	+	+	+
Pseudomonas fluorescens 11A1	Fillet line	Yellowish	R	+	+	+
Pseudomonas fluorescens 11B3	Surimi stuffer	Beige	R	+	+	+
Pseudomonas fluorescens 12A2	Salmon paté	Beige	R	+	+	+
Pseudomonas putida	ATCC 12633	Yellowish	R	+	+	+
Pseudomonas putida 7E4	Smoked salmon	Beige	R	+	+	+
Pseudomonas putida 12A3	Frozen salmon paté	Beige	R	+	+	+
Psychrobacter immobilis	ATCC 43116	Translucent	C-B	–	+	+
Serratia fonticola 2D3	Pollock surimi	Beige	R	+	+	–
Serratia marcescens	ATCC 13880	Pink	R	+	+	–
Shewanella putrefaciens	ATCC 8071	Yellowish	R	+	+	+
Shewanella putrefaciens	ATCC 49138	Yellowish	R	+	+	+

(C) coccus, (R) Rods, (C-B) coccobacilli

Table 2.2. Confirmation of identities of FITCCC strains to genus and species by Sherlock MIS

Strain	Genus No. ID	Species	
		No. ID	Mean SMI (± s.d.)
Alteromonas sp. 8C2	5	5	0.689 ± 0.031
Arthrobacter sp. 5B6	5	5	0.659 ± 0.071
B. subtilis 5D1	5	5	0.907 ± 0.05
C. freundii 4E5	4	4	0.653 ± 0.088
E. cloacae 12D5	5	3	0.777 ± 0.001
Flavobacterium sp. 5C6	5	5	0.620 ± 0.034
H. alvei 2F1	3	3	0.716 ± 0.057
H. alvei 12E1	4	4	0.725 ± 0.171
M. varians 7F1	5	0	
M. morganii 2E5	5	5	0.535 ± 0.134
Moraxella sp. 5E6	6	6	0.533 ± 0.082
P. alcalifaciens 7F2	4	0	
P. fluorescens 2A1	5	5	0.740 ± 0.180
P. fluorescens 11A1	5	4	0.535 ± 0.057
P. fluorescens 11B3	7	3	0.375 ± 0.022
P. fluorescens 12A2	5	1	0.422
P. putida 7E4	6	6	0.765 ± 0.115
P. putida 12A3	5	5	0.579 ± 0.184
S. fonticola 2D3	5	5	0.414 ± 0.221
S. xylosus 13A4	5	0	
S. faecium 6A4	5	5	0.777 ± 0.051

Table 2.3. Confirmation of identities of ATCC strains to genus and species by Sherlock MIS

Strain	Genus No. ID	Species	
		No. ID	Mean SMI (± s.d.)
A. hydrophila 35654	5	5	0.889 ± 0.023
E. coli 11303	5	5	0.744 ± 0.068
P. fluorescens 13525	5	5	0.874 ± 0.112
P. fluorescens 17574	5	5	0.709 ± 0.123
P. fluorescens 31419	5	3	0.624 ± 0.110
P. putida 12633	5	5	0.716 ± 0.047
P. nigrifaciens 19375	5	5	0.851 ± 0.046
P. immobilis 43116	5	5	0.900 ± 0.012
S. marcescens 13880	4	3	0.619 ± 0.063
S. putrefaciens 49138	5	5	0.955 ± 0.012
S. putrefaciens 8071	5	5	0.771 ± 0.043
S. epidermidis 14990	5	4	0.679 ± 0.079

Table 2.4. Comparison of identification capability of Sherlock MIS for the FITCCC and ATCC strains

Isolates	Total analyzed	Identification to Species*
FITC	108	73 %
ATCC	60	92 %

*Species level identification (%) = $\frac{\text{Total analysis – Species level ID}}{\text{Species level ID}} \times 100$

Table 2.5. Fatty Acid profiles of gram-positive bacteria from the FITCCC and ATCC *

Fatty Acids	*Arthro-bacter* sp. 5B6	*B. subtilis* 5D1	*M. varians* 7F1	*S. xylosus* 13A4	*S. epider-midis* 14990	*S. faecium* 6A4
14:0	1.82 ± 0.16	T	T	3.42 ± 0.31	2.09 ± 0.21	7.21 ± 0.37
16:0	1.77 ± 0.30	4.84 ± 0.93	1.62 ± 0.21	2.69 ± 0.16	6.33 ± 1.77	16.68 ±0.56
18:0	T	T	T	2.41 ± 0.40	14.67 ± 3.49	T
20:0	A	A	A	1.32 ±0.50	11.44 ± 3.36	A
19:0 cy ω8c	A	A	A	A	A	8.74 ±1.45
13:0 i	T	T	T	27.29 ± 0.94	2.76 ± 0.34	A
14:0 i	6.33 ± 1.23	T	1.34 ± 0.14	3.44 ± 0.22	5.16 ± 0.75	A
15:0 i	6.52 ± 3.10	23.68 ± 3.27	13.81 ± 0.33	13.81 ± 0.44	14.44 ± 1.66	A
16:0 i	3.05 ± 0.47	2.30 ± 0.12	8.48 ± 0.49	1.87 ± 0.13	1.06 ± 0.09	A
17:0 i	T	10.27 ± 0.47	2.13 ± 0.05	7.74 ± 0.24	3.35 ± 0.10	A
19:0 i	A	A	A	2.11 ± 0.42	1.48 ± 0.40	A
17:1 i ω10c	A	1.92 ± 0.28	A	T	A	A
13:0 ai	T	T	T	9.20 ± 0.58	T	A
15:0 ai	77.94 ± 4.03	38.72 ± 1.34	57.09 ± 0.18	16.89 ± 0.47	31.45 ± 4.64	A
17:0 ai	1.45 ± 0.26	11.47 ± 1.42	13.09 ± 0.20	2.52 ± 0.11	2.33 ± 0.18	A
16:1 ω11c	A	2.62 ± 0.21	A	T	A	A
18:1 ω7c	A	T	A	A	T	47.79 ± 2.07
SF 3	A	A	A	T	A	17.37 ± 0.52
SF 5	A	1.10 ± 0.11	A	A	A	A

Table 2.5. contd...

Fatty Acids	*Arthro-bacter* sp. 5B6	*B. subtilis* 5D1	*M. varians* 7F1	*S. xylosus* 13A4	*S. epider-midis* 14990	*S. faecium* 6A4
Groupings						
Sat	3.70 ± 0.45	6.10 ± 1.22	2.80 ± 0.41	10.88 ± 1.25	34.69 ± 6.29	25.11 ± 1.51
Sat i	16.09 ± 4.77	37.36 ± 3.29	25.98 ± 0.61	58.03 ± 0.92	28.67 ± 2.30	A
Sat ai	79.83 ± 4.26	50.21 ± 2.50	70.59 ± 1.35	29.28 ± 0.92	34.87 ± 4.55	A
Cy	A	A	A	A	A	8.74 ± 1.45
Unsat	A	2.72 ± 0.33	T	1.10 ± 0.33	T	65.99 ± 1.52
Unsat i	A	1.92 ± 0.28	A	A	T	A
*Totals***						
Branched	96.14 ± 0.52	89.53 ± 1.32	96.57 ± 0.76	87.87 ± 1.38	63.98 ± 6.48	T
Saturated	99.72 ± 0.06	93.76 ± 0.36	99.77 ± 0.25	98.59 ± 0.37	99.45 ± 0.69	34.01 ± 1.52
Unsatur-ated	A	5.14 ± 0.33	T	1.35 ± 0.40	T	65.99 ± 1.52

*Fatty acid are >1% (total of fatty acids) , T denotes trace amounts <1% and A denotes below detection.

** Totals based on average ± s.d. of all replicates and not on the sum of average fatty acids

Key: (i) iso, (ai) anteiso, (cy) cyclopropane, (Sat) saturated straight chain, (Sat i) saturated iso, (Sat ai)- saturated anteiso, (Cy)- Cyclopropane, (Unsat) unsaturated straight chain, (Unsat ai) unsaturated anteiso, Branched = Sat i + Sat ai + Unsat i, Saturated = Sat + Sat i + Sat ai + Cy, Unsaturated = Unsat + Unsat i.

Refer Material and Methods (p. 73) for explanation of SF 3 and 5.

Table 2.6. Fatty acid profiles of gram-negative, oxidase-negative bacteria from the FITCCC and ATCC*

Fatty Acids	*C. freundii* 4E5	*E. cloacae* 12D5	*E. coli* 1D3	*H. alvei* 12E1	*H. alvei* 2F1	*S. fonticola* 2D3	*S. marce-scens* 13880
12:0	3.64 ± 0.76	2.61 ± 0.12	3.75 ± 0.15	3.83 ± 0.22	3.94 ± 0.42	2.43 ± 0.11	2.22 ± 0.26
14:0	6.70 ± 0.42	7.28 ± 0.21	5.64 ± 0.26	7.45 ± 0.46	6.28 ± 0.41	5.79 ± 0.18	6.15 ± 0.38
15:0	1.12 ± 0.04	2.71 ± 0.26	T	1.81 ± 0.76	2.09 ± 0.47	5.53 ± 2.92	T
16:0	24.02 ± 1.58	26.57 ± 0.75	27.32 ± 0.88	33.36 ± 0.68	33.07 ± 0.56	28.15 ± 0.92	28.24 ± 0.81
17:0	T	1.71 ± 0.12	T	T	T	1.35 ± 0.52	T
14:0 2-OH	A	T	A	A	A	A	1.89 ± 0.20
17:0 cy	1.64 ± 0.25	8.64 ± 1.71	9.84 ± 3.06	17.63 ± 5.31	21.57 ± 5.11	12.25 ± 2.78	7.77 ± 3.00
19:0 cy ω8c	T	T	3.39 (1.35)	T	T	T	1.47 ± 0.60
18:1 ω7c	16.56 ± 1.73	21.28 ± 1.03	28.61 ± 3.01	7.47 ± 1.11	7.44 ± 0.89	8.12 ± 0.59	17.01 ± 1.18
SF 2	9.82 ± 1.45	8.52 ± 0.55	8.37 ± 0.54	8.96 ± 0.69	9.21 ± 1.25	8.74 ± 0.56	9.86 ± 0.69
SF 3	33.25 ± 0.87	16.18 ± 3.01	8.96 ± 2.73	14.88 ± 5.07	10.63 ± 2.99	21.79 ± 6.38	19.11 ± 2.82

Table 2.6. contd...

Fatty Acids	*C. freundii* 4E5	*E. cloacae* 12D5	*E. coli* 1D3	*H. alvei* 12E1	*H. alvei* 2F1	*S. fonticola* 2D3	*S. marce-scens* 13880
Groupings							
Sat	36.28 ± 0.46	41.81 ± 1.42	38.62 ± .22	47.70 ± 1.58	47.04 ± 1.26	44.74 ± 3.44	38.68 ± 0.64
Sat OH	T	T	T	T	T	T	3.62 ± 0.30
Sat i	A	A	T	A	A	T	T
Sat ai	A	A	T	T	T	A	T
Cy	1.92 ± 0.32	9.12 ± 1.91	13.23 ± 4.40	17.90 ± 5.40	22.08 ± 5.33	12.33 ± 2.85	9.24 ± 3.58
Unsat	41.55 ± 1.26	37.94 ± 4.01	37.98 ± 5.51	22.89 ± 6.08	18.81 ± 3.85	30.73 ± 6.87	36.38 ± 3.83
Unsat ai	T	A	A	A	A	A	A
Unsat OH	A	T		T	T	T	A
*Totals***							
Branched	T	A	T	T	A	A	T
Saturated	38.24 ± 0.74	51.24 ± 3.34	52.00 ± 5.56	65.79 ± 6.43	69.27 ± 4.86	57.79 ± 6.13	51.85 ± 4.05
Unsaturated	50.19 ± 1.26	38.01 ± 4.03	37.98 ± 5.51	22.94 ± 6.03	18.86 ± 3.81	30.92 ± 7.03	36.38 ± 3.83

*Fatty acids are >1%, T denotes trace amounts <1% and A denotes below detection.
** Totals based on average ± s.d. of all replicates and not on the sum of average fatty acids
Refer to Table 2.5 footnote for key to abbreviations, with the following inclusions: (Sat OH) saturated straight chain hydroxyl, (Unsat OH) unsaturated straight chain hydroxyl, Branched = Sat i + Sat ai + Unsat ai, Saturated = Sat + Sat OH + Sat i + Sat ai + Cy, Unsaturated = Unsat + Unsat OH + Unsat ai
Refer to Material and Methods (p. 73) for explanation of SF 2 and 3.

Table 2.7. Fatty acid profiles of gram-negative, oxidase-positive bacteria from the FITCCC and ATCC*

Fatty Acids	*Aero-monas hydro-phila* 35654	*Altero-*monas sp. 8C2	*Shewa-nella. putri-faciens* 49138	*Shewa-nella putri-faciensf* 8071	*Pseudo-altero-monas nigri-*faciens 19375	*Flavo-bacte-rium* sp. 5C6	*Morax-ella* sp. 5E6	*Psychro-bacter immo-bilis* 43116	*Provi-dencia. alcali-faciens* 7F2	*Morgan-ella. morg-anii* 2E5
10:0	A	A	A	A	A	A	1.94 ± 0.66	3.01 ± 0.24	A	A
12:0	4.57 ± 0.44	3.05 ± 0.51	1.26 ± 0.21	2.59 ± 0.41	2.61 ± 0.23	A	2.15 ± 0.65	A	T	4.36 ± 0.36
13:0	T	1.79 ± 0.30	1.66 ± 0.15	1.50 ± 0.17	T	A	A	A	T	T
14:0	3.29 ± 0.33	1.74 ± 0.18	T	1.57 ± 0.22	2.01 ± 0.11	T	T	T	5.69 ± 0.08	6.56 ± 0.33
15:0	T	7.25 ± 1.15	9.15 ± 1.44	6.39 ± 0.67	8.76 ± 0.19	T	T	T	3.50 ± 1.07	1.65 ± 0.11
16:0	17.29 ± 0.50	9.11 ± 0.93	4.67 ± 0.50	6.89 ± 0.65	16.13 ± 0.68	T	3.32 ± 0.52	1.18 ± 0.18	28.56 ± 0.48	32.21 ± 1.11
17:0	T	2.33 ± 0.30	2.68 ± 0.34	1.50 ± 0.26	3.12 ± 0.22	A	T	T	T	T
18:0	T	T	T	T	T	A	3.29 ± 0.61	3.55 ± 0.60	T	T
20:0	A	A	A	A	A	A	A	A	A	A
11:0 2-OH	A	A	A	A	2.08 ± 0.21	A	A	A	A	A
12:0 3-OH	A	2.39 ± 0.53	1.42 ± 0.26	2.55 ± 0.44	7.18 ± 0.76	A	4.60 ± 1.70	3.52 ± 0.33	T	T
16:0 3-OH	T	A	A	A	A	4.22 ± 0.49	A	A	T	T

Table 2.7. contd...

Fatty Acids	*Aero-monas hydro-phila* 35654	*Altero-*monas sp. 8C2	*Shewa-nella. putri-faciens* 49138	*Shewa-nella putri-faciens* 8071	*Pseudo-altero-monas nigri-faciens* 19375	*Flavo-bacte-rium* sp. 5C6	*Morax-ella* sp. 5E6	*Psychro-bacter immo-bilis* 43116	*Provi-dencia. alcali-faciens* 7F2	*Morgan-ella morg-anii* 2E5
17:0 cy	A	A	A	A	A	A	A	A	A	18.26 ± 3.05
19:0 cy ω8c	A	A	A	T	A	A	A	A	T	4.29 ± 0.86
13:0 i	T	6.52 ± 0.64	5.48 ± 0.25	7.91 ± 0.70	T	T	A	A	A	A
14:0 i	T	2.12 ± 0.17	T	T	A	T	A	A	A	A
15:0 i	1.34 ± 0.30	10.17 ± 0.64	23.41 ± 0.88	15.69 ± 1.46	T	52.57 ± 2.77	T	T	A	A
16:0 i	T	T	T	T	T	T	A	A	A	A
17:0 i	1.65 ± 0.56	T	T	T	T	T	4.43 ± 1.14	1.66 ± 0.11	A	T
19:0 i	A	A	A	A	A	A	T	1.19 ± 0.19	A	A
12:0 i 3-OH	A	T	A	A	1.20 ± 0.14	A	A	A	A	A
13:0 i 3-OH	A	T	3.17 ± 0.24	2.56 ± 0.44	T	T	T	T	A	A
15:0 i 3-OH	1.26 ± 0.35	T	T	T	A	6.41 ± 0.72	A	A	A	A
17:0 i 3-OH	A	A	A	A	A	10.92 ± 1.64	A	A	A	A

Table 2.7. contd...

Fatty Acids	*Aero-monas hydro-phila* 35654	*Altero-*monas sp. 8C2	*Shewa-nella. putri-faciens* 49138	*Shewa-nella putri-faciens* 8071	*Pseudo-altero-monas nigri-faciens* 19375	*Flavo-bacte-rium* sp. 5C6	*Morax-ella* sp. 5E6	*Psychro-bacter immo-bilis* 43116	*Provi-dencia alcali-facie-ns* 7F2	*Morgan-ella morg-anii* 2E5
15:0 ai	A	T	T	T	T	1.91 ± 0.39	A	A	A	A
15:0 ai	A	T	T	T	T	1.91 ± 0.39	A	A	A	A
17:0 ai	T	A	A	T	T	A	A	T	T	A
15:1ω8c	T	T	1.21 ± 0.08	1.74 ± 0.12	5.69 ± 0.22	A	T	T	T	A
16:1 ω9c	A	T	1.22 ± 0.17	1.72 ± 0.06	A	A	A	A	A	A
17:1 ω6c	T	1.04 ± 0.18	1.19 ± 0.22	1.10 ± 0.16	A	A	A	A	T	A
17:1 ω8c	1.04 ± 0.32	13.02 ± 1.84	20.14 ± 2.00	14.21 ± 1.19	11.43 ± 0.72	A	9.91 ± 1.53	8.64 ± 1.70	T	T
18:1 ω7c	18.77 ± 0.53	2.92 ± 0.45	1.65 ± 0.26	2.39 ± 0.30	A	A	A	A	11.99 ± 0.26	8.69 ± 1.59
18:1 ω9c	A	1.85 ± 0.26	1.53 ± 0.23	1.41 ± 0.07	T	T	48.37 ± 1.74	63.04 ± 2.25	A	A
18:3 ω6c (6,9,12)	A	A	A	A	A	A	T	1.94 ± 0.19	A	A
SF 1	A	2.16 ± 0.35	2.70 ± 0.16	2.52 ± 0.18	1.18 ± 0.19	T	T	T	A	A
SF 2	6.67 ± 0.97	2.90 ± 0.55	T	1.80 ± 0.31	T	T	T	1.09 ± 0.14	8.07 ± 0.51	9.62 ± 0.92

Table 2.7. contd...

Fatty Acids	*Aeromonas hydrophila* 35654	*Alteromonas* sp. 8C2	*Shewanella. putrifaciens* 49138	*Shewanella putrifaciens* 8071	*Pseudoalteromonas nigrifaciens* 19375	*Flavobacterium* sp. 5C6	*Moraxella* sp. 5E6	*Psychrobacter immobilis* 43116	*Providencia alcalifaciens* 7F2	*Morganella morganii* 2E5
SF 3	36.35 ± 1.59	20.51 ± 1.31	10.03 ± 1.25	18.60 ± 0.80	30.33 ± 0.99	2.20 ± 0.30	16.13 ± 1.08	3.99 ± 0.15	37.39 ± 0.81	8.97 ± 1.56
SF 4	1.49 ± 0.48	A	T	A	A	16.84 ± 1.30	T	A	A	A
SF 7	A	A	A	A	A	A	T	1.04 ± 0.13	A	A
Groupings										
Sat	27.58 ± 0.63	25.87 ± 0.49	20.98 ± 1.86	20.89 ± 1.11	33.98 ± 0.68	1.51 ± 0.13	14.31 ± 3.93	10.26 ± 1.01	39.00 ± 1.17	46.50 ± 1.17
Sat OH	T	3.17 ± 0.64	3.45 ± 0.42	2.43 ± 0.23	9.78 ± 1.01	4.91 ± 0.62	4.78 ± 1.50	4.10 ± 0.29	T	T
Sat i	4.39 ± 1.30	19.90 ± 0.69	25.16 ± 2.12	30.54 ± 1.15	2.32 ± 0.11	53.49 ± 2.92	5.42 ± 1.38	3.34 ± 0.29	T	T
Sat i OH	1.26 ± 0.35	2.67 ± 0.43	3.17 ± 0.46	3.92 ± 0.24)	2.00 ± 0.19	17.89 ± 2.19	T	T	A	T
Sat ai	T	T	T	T	T	1.91 ± 0.39	A	T	A	A
Cy	A	A	T	A	A	A	A	A	A	22.55 ± 3.77
Unsat	56.51 ± 0.88	42.36 ± 1.76	42.17 ± 0.92	37.84 ± 0.50	49.45 ± 1.14	T	75.29 ± 2.10	78.44 ± 1.10	13.42 ± 0.44	18.33 ± 3.22

Table 2.7. contd...

Fatty Acids	*Aero-monas hydro-phila* 35654	*Altero-*monas sp. 8C2	*Shewa-nella. putri-faciens* 49138	*Shewa-nella putri-faciens* 8071	*Pseudo-altero-monas nigri-faciens* 19375	*Flavo-bacte-rium* sp. 5C6	*Morax-ella* sp. 5E6	*Psychro-bacter immo-bilis* 43116	*Provi-dencia alcali-facie-ns* 7F2	*Morgan-ella morg-anii* 2E5
*Totals***										
Branched	6.06 ± 1.69	22.76 ± 1.01	28.71 ± 2.32	34.87 ± 1.25	4.96 ± 0.20	73.56 ± 1.71	5.57 ± 1.42	3.57 ± 0.42	T	T
Saturated	34.18 ± 1.32	51.80 ± 1.14	53.07 ± 0.78	58.04 ± 0.41	48.69 ± 1.06	79.71 ± 1.74	24.59 ± 4.02	18.15 ± 1.12	39.38 ± 1.11	69.36 ± 3.35
Unsatura-ted	56.51 ± 0.88	42.36 ± 1.76	42.28 ± 1.07	38.00 ± 0.50	49.50 ± 1.17	T	75.30 ± 2.10	78.44 ± 1.10	13.50 ± 0.57	18.40 ± 3.36

*Fatty acids are >1%, T denotes trace amounts <1% and A denotes below detection.

** Totals based on average ± s.d. of all replicates and not on the sum of average fatty acids given in table

Refer to Table 2.5 and 2.6 footnotes for key to abbreviations, with the following inclusions: (Sat i OH) saturated iso hydroxyl, Branched = Sat i + Sat i OH + Sat ai +, Saturated = Sat + Sat OH + Sat i + Sat i OH + Sat ai + Cy, Unsaturated = Unsat only. Refer to Material and Methods (pg. 73) for detailed explanation of SF 1, 2, 3, 4 and 7.

Table 2.8. Fatty acid profiles of gram-negative, oxidase-positive bacteria belonging to genus *Pseudomonas* from the FITCCC and ATCC *

Fatty Acids	*Pseudo-monas fluore-scens* 11A1	*Pseudo-monas fluore-scens* 11B3	*Pseudo-monas fluore-scens* 12A2	*Pseudo-monas fluore-scens* 2A1	*Pseudo-monas fluore-scens* 17574
12:0	1.59 ± 0.09	2.39 ± 0.34	1.89 ± 0.19	3.33 ± 0.19	3.51 ± 0.71
16:0	30.11 ± 0.39	24.29 ± 0.82	32.14 ± 1.51	27.37 ± 0.82	26.83 ± 1.40
10:0 3-OH	3.90 ± 0.53	4.01 ± 0.76	4.01 ± 0.48	4.02 ± 0.36	4.68 ± 0.64
12:0 2-OH	6.45 ± 0.29	5.93 ± 1.09	5.54 ± 0.68	4.85 ± 0.44	5.11 ± 0.87
12:0 3-OH	5.37 ± 0.58	5.79 ± 0.96	4.78 ± 0.42	5.08 ± 0.31	5.16 ± 0.85
17:0 cy	6.89 ± 2.63	4.18 ± 1.78	8.78 ± 1.54	1.46 ± 0.93	T
17:1 ω7c	A	A	T	A	A
18:1 ω7c	11.80 ± 0.47	20.23 ± 1.91	8.51 ± 0.77	14.97 ± 0.47	15.37 ± 1.33
SF 3	31.03 ± 2.61	30.08 ± 1.71	30.45 ± 0.97	36.38 ± 0.63	36.54 ± 0.72
Groupings					
Sat	33.43 ± 0.48	28.33 ± 0.77	35.70 ± 1.37	32.36 ± 0.70	32.12 ± 0.66
Sat OH	15.72 ± 1.33	15.93 ± 2.66	14.35 ± 1.53	A	14.94 ± 2.35
Cy	6.92 ± 2.66	4.58 ± 1.95	9.74 ± 1.69	1.46 ± 0.93	T
Unsat	43.16 ± 2.33	50.65 ± 2.28	39.42 ± 1.20	51.73 ± 0.88	52.25 ± 1.96
*Totals***					
Saturated	56.36 ± 2.18	48.97 ± 8.17	60.09 ± 1.20	47.89 ± 1.11	47.63 ± 1.98
Unsaturated	43.17 ± 2.33	50.81 ± 2.27	39.44 ± 1.23	51.87 ± 1.00	52.27 ± 1.94

Table 2.8. contd...

Fatty Acids	*Pseudo-monas fluore-scens* 31419	*Pseudo-monas fluore-scens* 13525	*Pseudo-monas putida* 12633	*Pseudo-monas putida* 7E4	*Pseudo-monas putida* 12A3
12:0	1.36 ± 0.15	2.85 ± 0.31	3.15 ± 0.26	1.95 ± 0.22	3.60 ± 0.12
16:0	29.20 ± 1.49	28.19 ± 1.26	25.73 ± 0.72	29.62 ± 0.67	27.66 ± 1.70
10:0 3-OH	4.71 ± 0.84	4.32 ± 1.36	4.40 ± 0.33	3.25 ± 0.26	4.74 ± 0.23
12:0 2-OH	6.12 ± 0.96	4.72 ± 0.54	5.20 ± 0.44	5.85 ± 0.30	4.93 ± 0.54
12:0 3-OH	5.14 ± 0.77	4.69 ± 0.59	5.04 ± 0.37	4.83 ± 0.41	5.19 ± 0.21
17:0 cy	4.95 ± 0.22	T	7.51 ± 2.88	10.75 ± 2.52	7.50 ± 3.68
17:1 ω7c	A	A	A	17.67 ± 0.62	A
18:1 ω7c	15.29 ± 0.83	18.92 ± 0.79	18.04 ± 0.71	A	10.68 ± 1.23
SF 3	29.69 ± 1.93	33.16 ± 0.72	28.21 ± 3.07	22.57 ± 2.45	32.23 ± 3.62
Groupings					
Sat	32.53 ± 1.33	32.49 ± 1.18	30.47 ± 0.83	33.51 ± 0.62	33.17 ± 1.78
Sat OH	15.97 ± 2.54	13.86 ± 2.42	14.64 ± 1.04	14.01 ± 0.98	14.87 ± 0.78
Cy	5.14 ± 2.15	T	7.71 ± 3.05	11.20 ± 2.62	8.41 ± 4.27
Unsat	45.30 ± 1.47	52.46 ± 1.13	46.55 ± 2.87	40.58 ± 2.81	43.14 ± 4.33
*Totals***					
Saturated	53.91 ± 1.43	47.32 ± 1.20	52.94 ± 2.83	58.42 ± 2.78	56.64 ± 4.19
Unsaturated	45.50 ± 1.48	52.52 ± 1.14	46.60 ± 2.84	40.57 ± 2.81	43.14 ± 4.33

*Fatty acids are >1%, T denotes trace amounts <1% and A denotes either absent or below detection. ** Totals based on average ± s.d. of all replicates and not on the sum of average fatty acids given in table. Refer to Table 2.5. footnote for key to abbreviations, Saturated = Sat + Sat OH + Cy, Unsaturated = Unsat only
Refer to Material and Methods (pg. 73) for detailed explanation of SF 3.

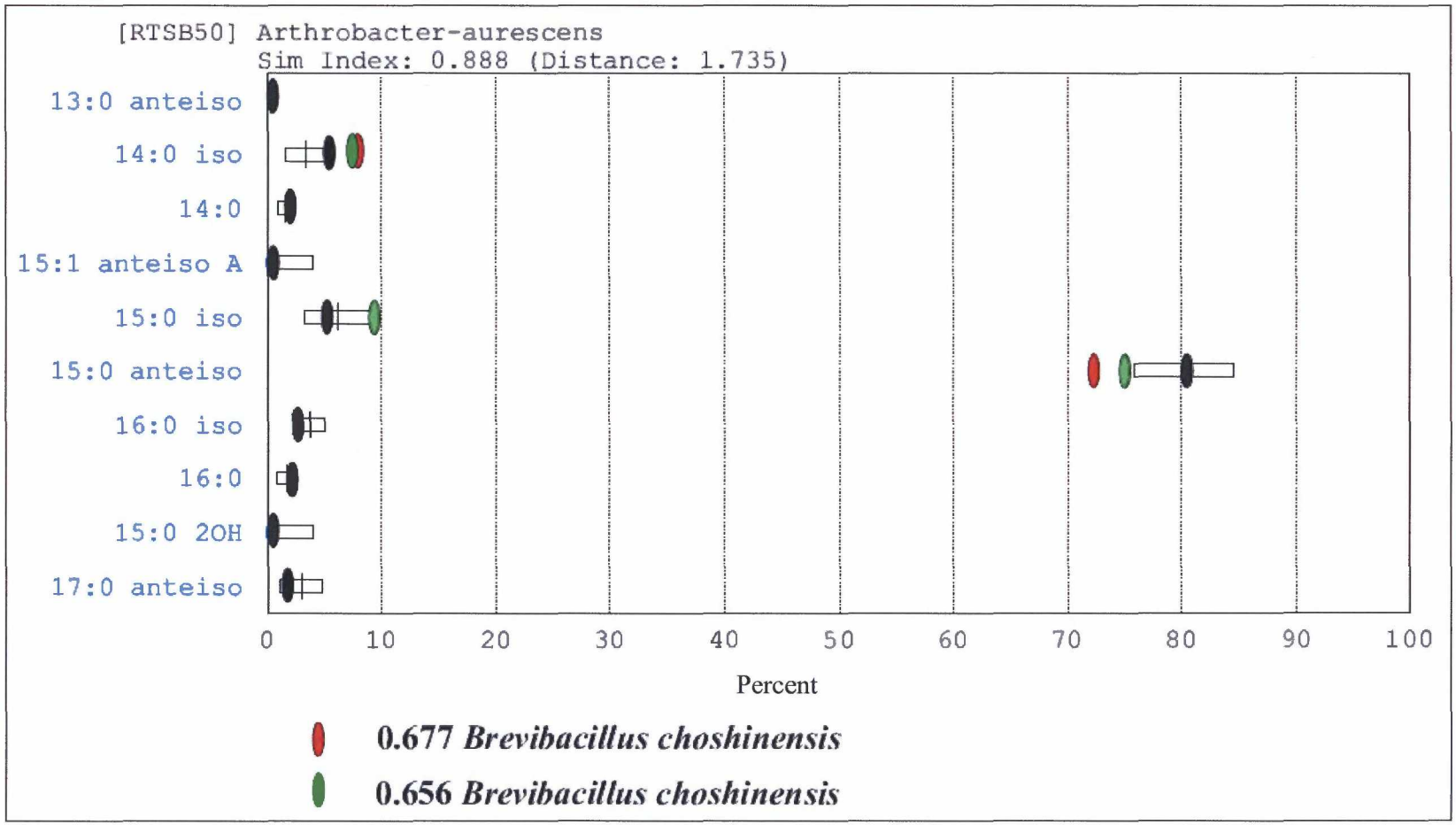

Figure 2.1. Comparison chart showing the deviation in fatty acids leading to the identification of two replicates of *Arthrobacter* sp. 5B6 as *Brevibacillus choshinensis* (first rank).

Key to comparison chart: [RTSB50] is the fatty acid library of aerobes grown on TSBA at 28°C for 24h. *Arthrobacter aurescens*: Identification of the test organism given by the Sherlock MIS with Sim Index (Similarity Index) of 0.888. Distance: The Euclidean distance of the current fatty acid profile (marked in black) to the library entry. Values on X axis are in percent.

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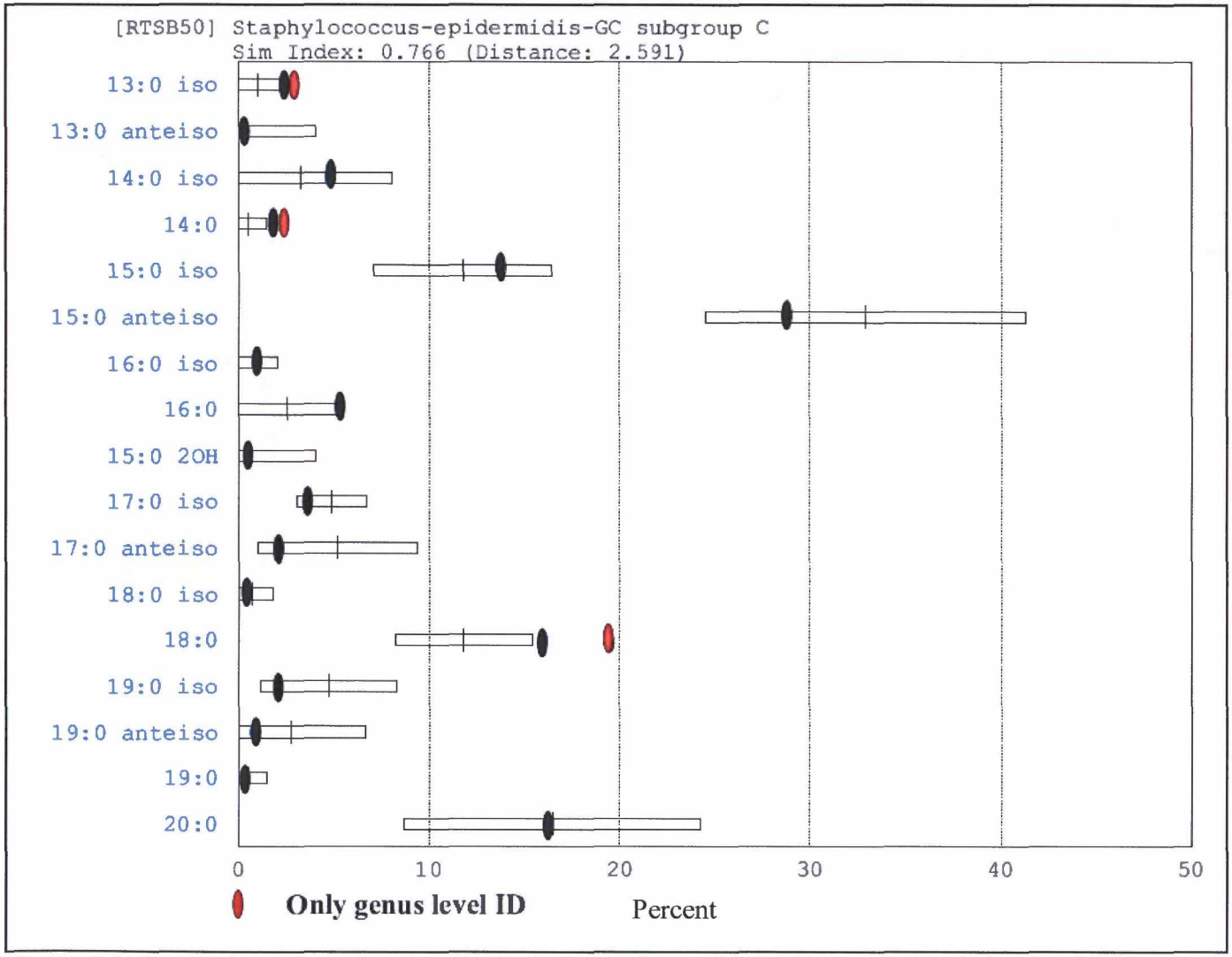

Figure 2.2. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of three replicates of *Staphylococcus epidermidis* ATCC 14990.

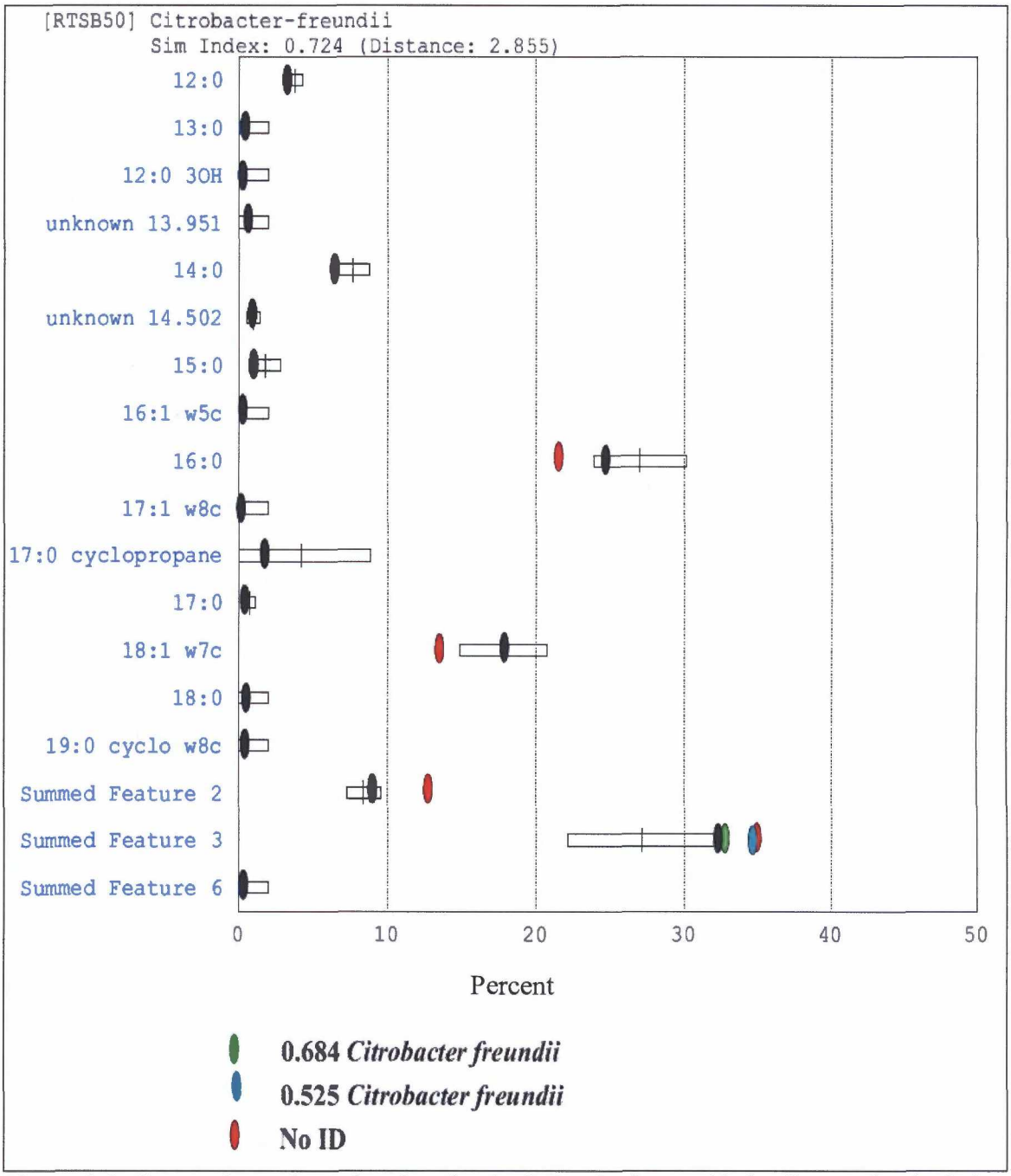

Figure 2.3. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of three replicates of *Citrobacter freundii* 4E5.

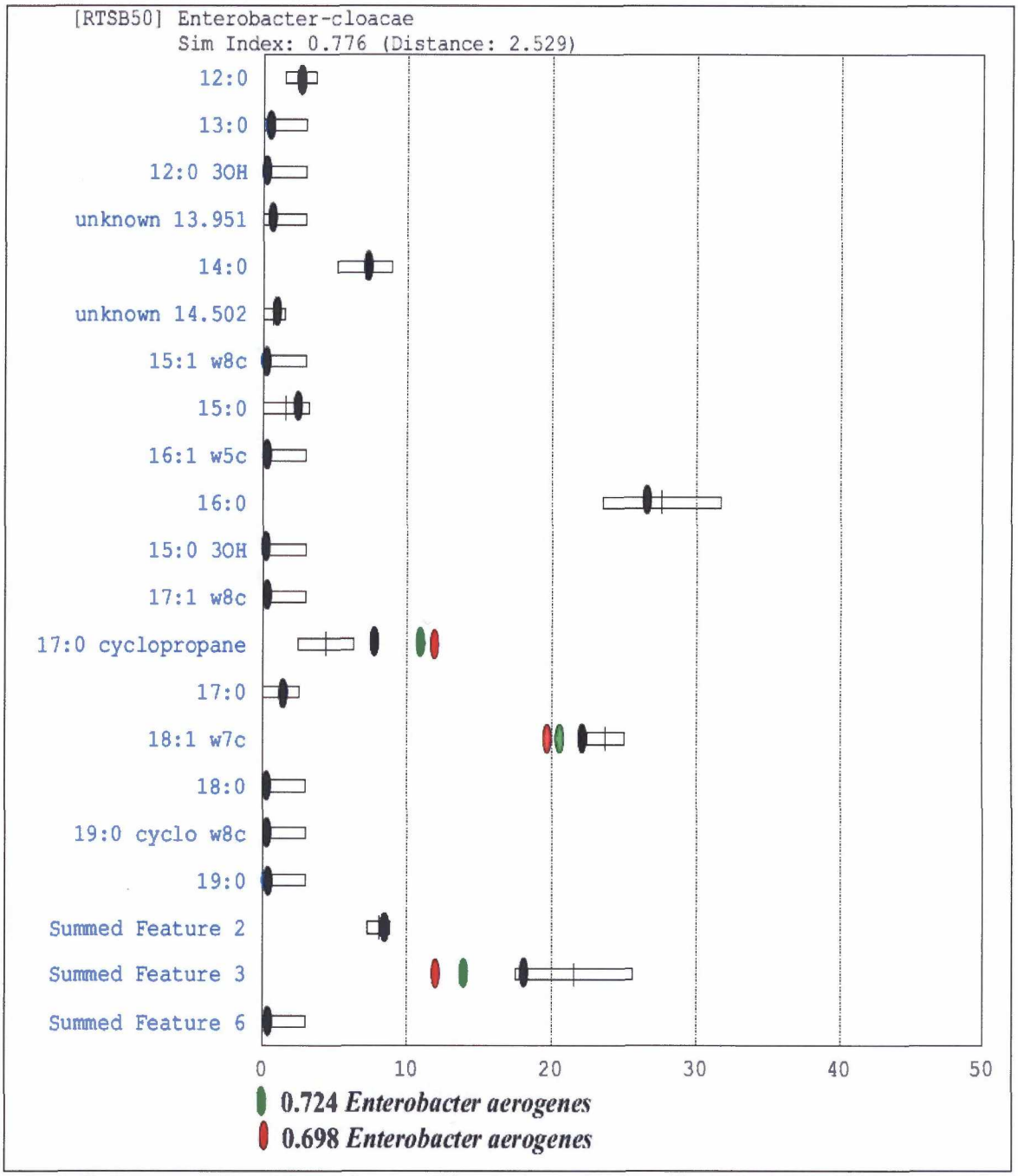

Figure 2.4. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of two replicates of *Enterobacter cloacae* 12D5

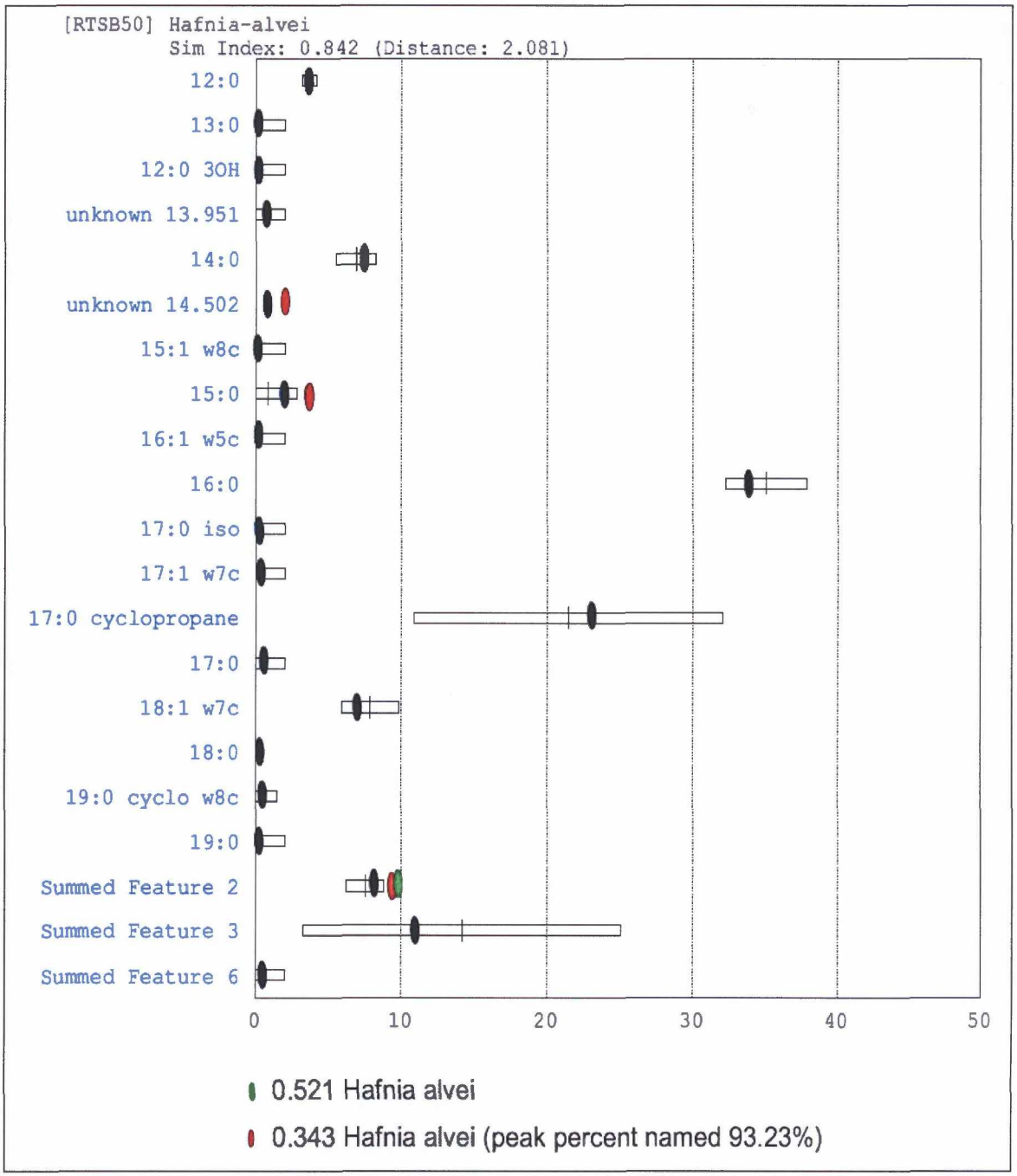

Figure 2.5. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of two replicates of *Hafnia alvei* 12E1.

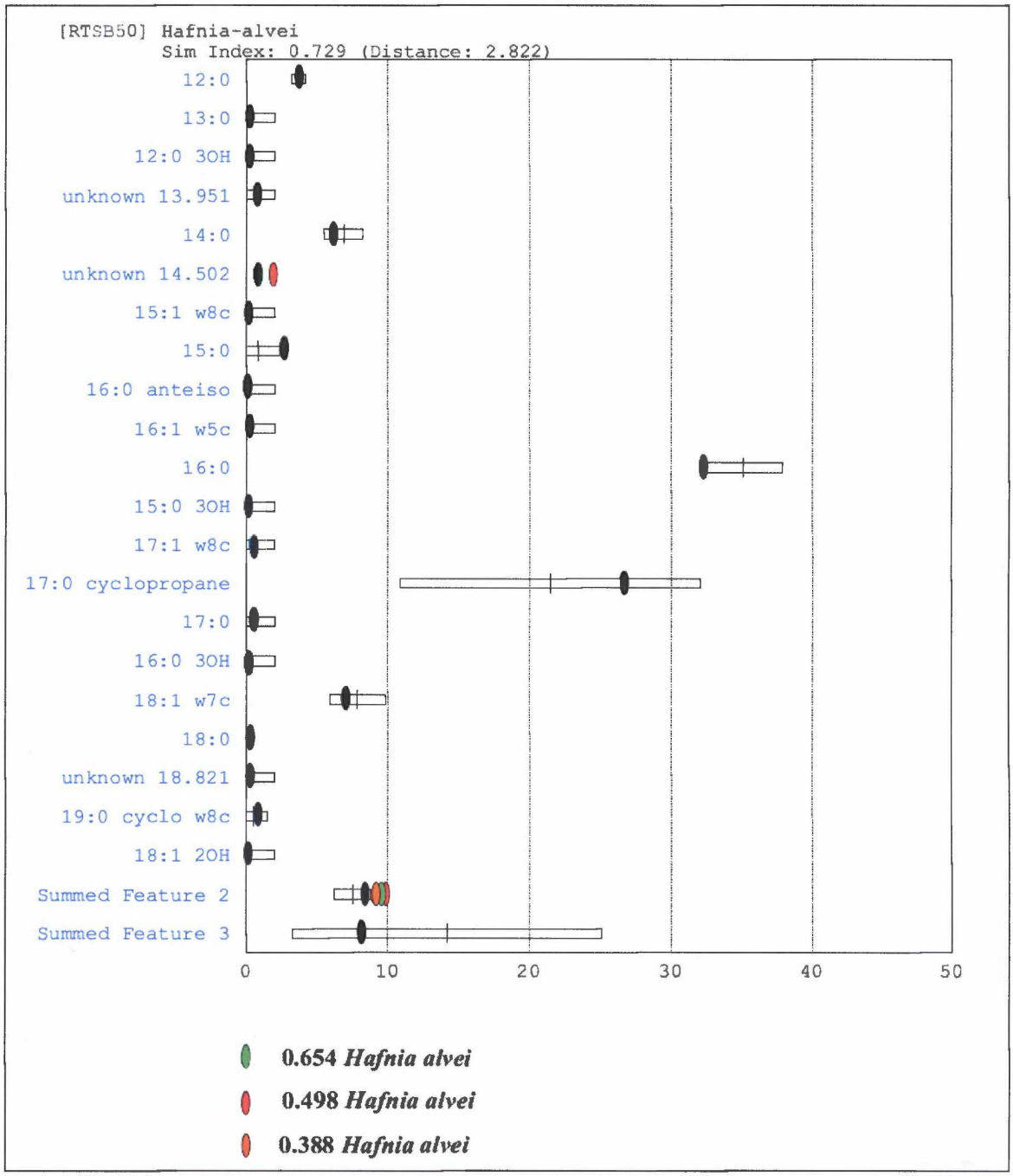

Figure 2.6. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of three replicates of *Hafnia alvei* 2F1.

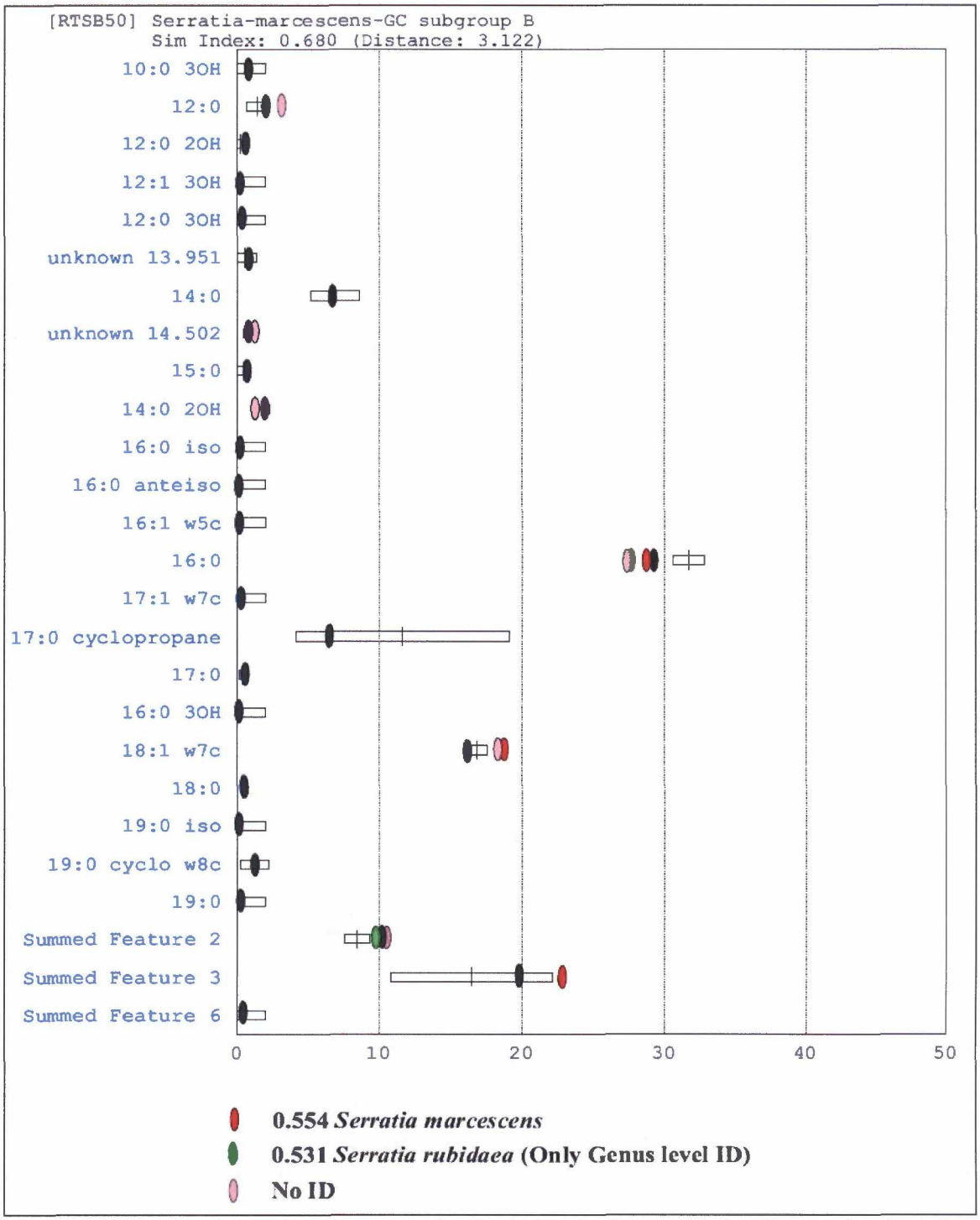

Figure 2.7. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of three replicates of *Serratia marcescens* ATCC 13880.

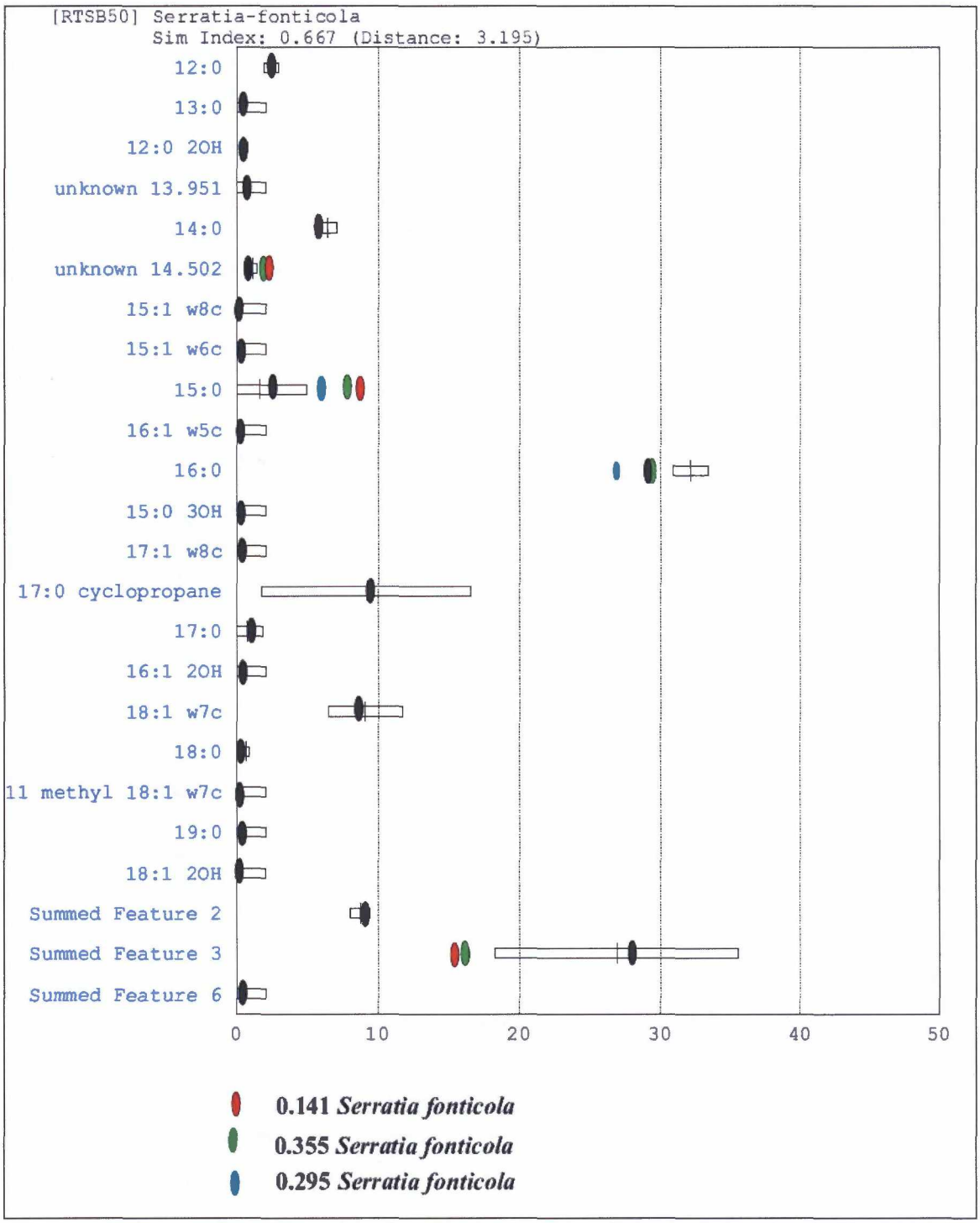

Figure 2.8. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of three replicates of *Serratia fonticola* 2D3.

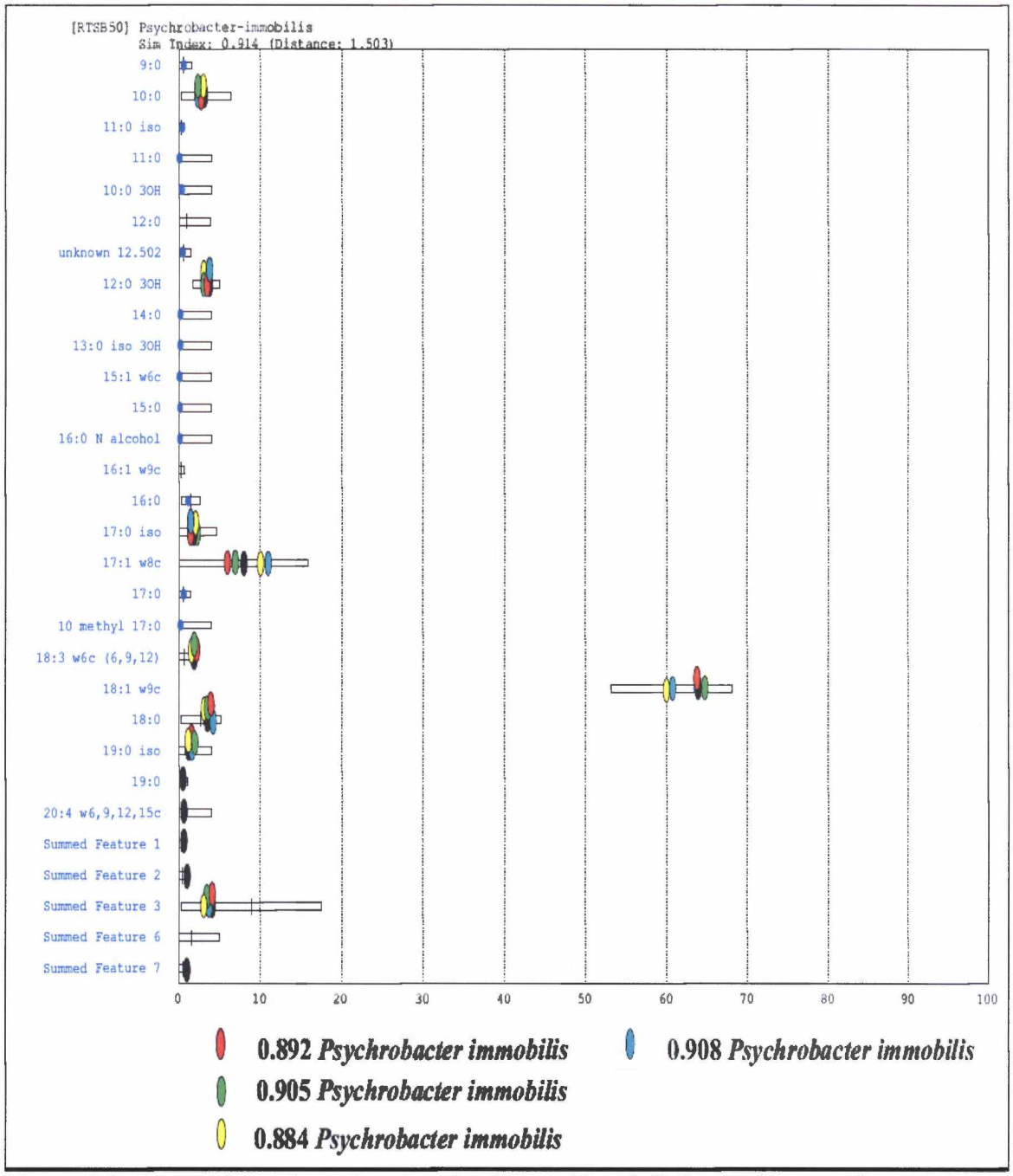

Figure 2.9. Comparison chart showing the fatty acids of all five replicates of *Psychrobacter immobilis* ATCC 43116.

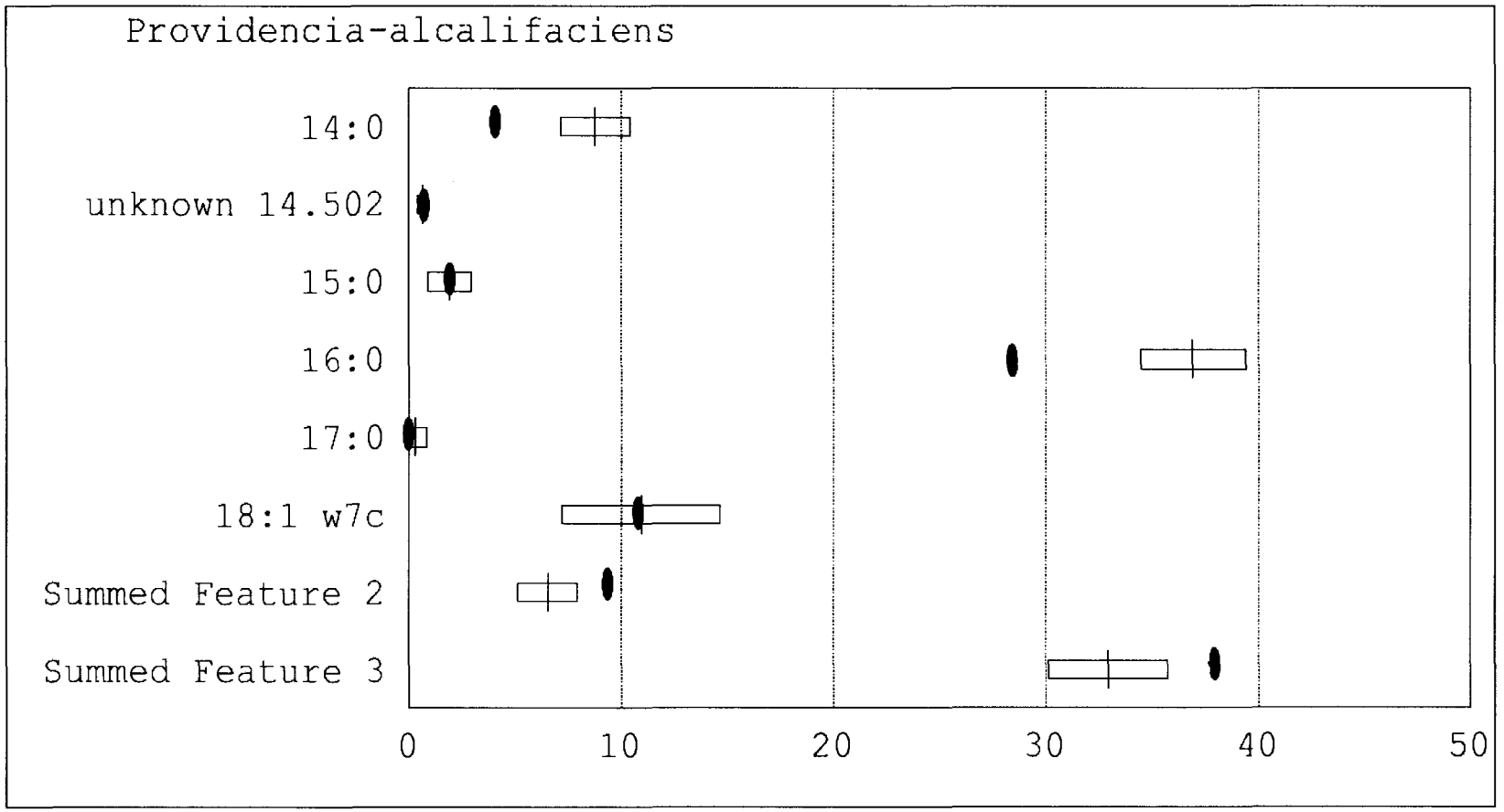

Figure 2.10. Comparison chart showing the deviation in average fatty acid profile (filled ovals) of *Providencia alcalifaciens* 7F2 from the mean of respective fatty acids in the RTSB50 library entry of *P. alcalifaciens*.

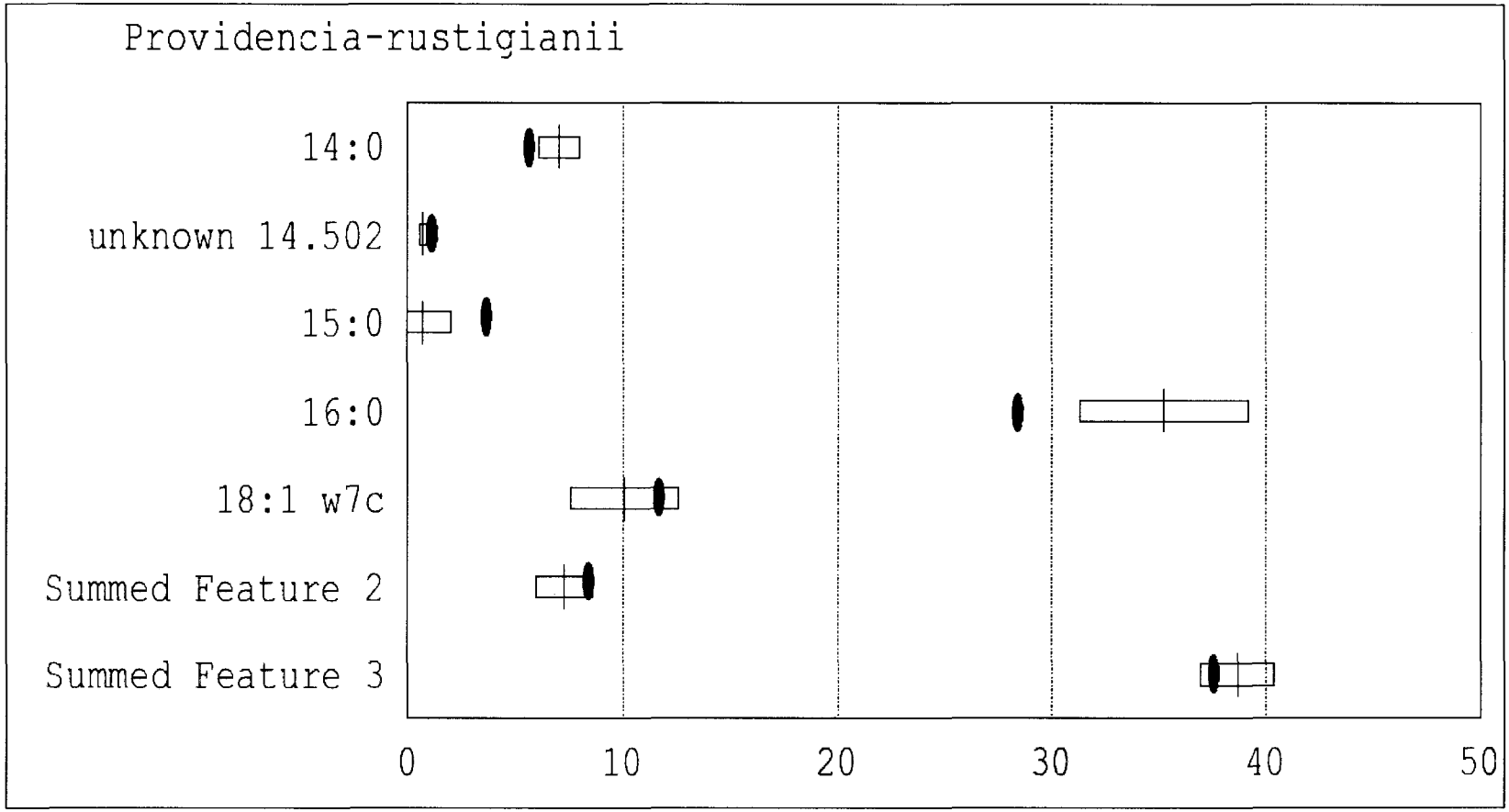

Figure 2.11. Comparison chart showing the deviation in average fatty acid profile (filled ovals) of *Providencia alcalifaciens* 7F2 from the mean of respective fatty acids in the RTSB50 library entry of *P. rustigianii*.

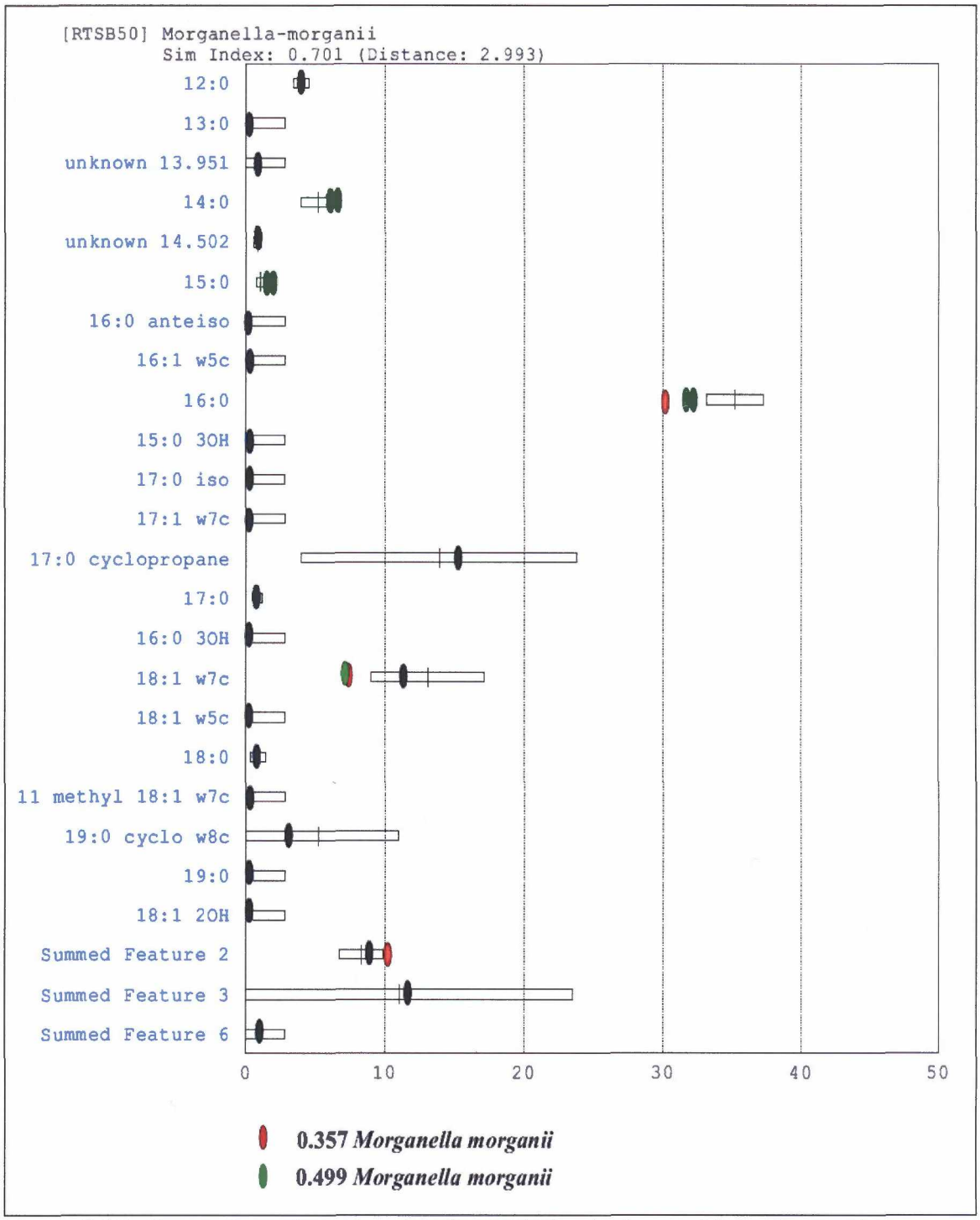

Figure 2.12. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of two replicates of *Morganella morganii* 2E5.

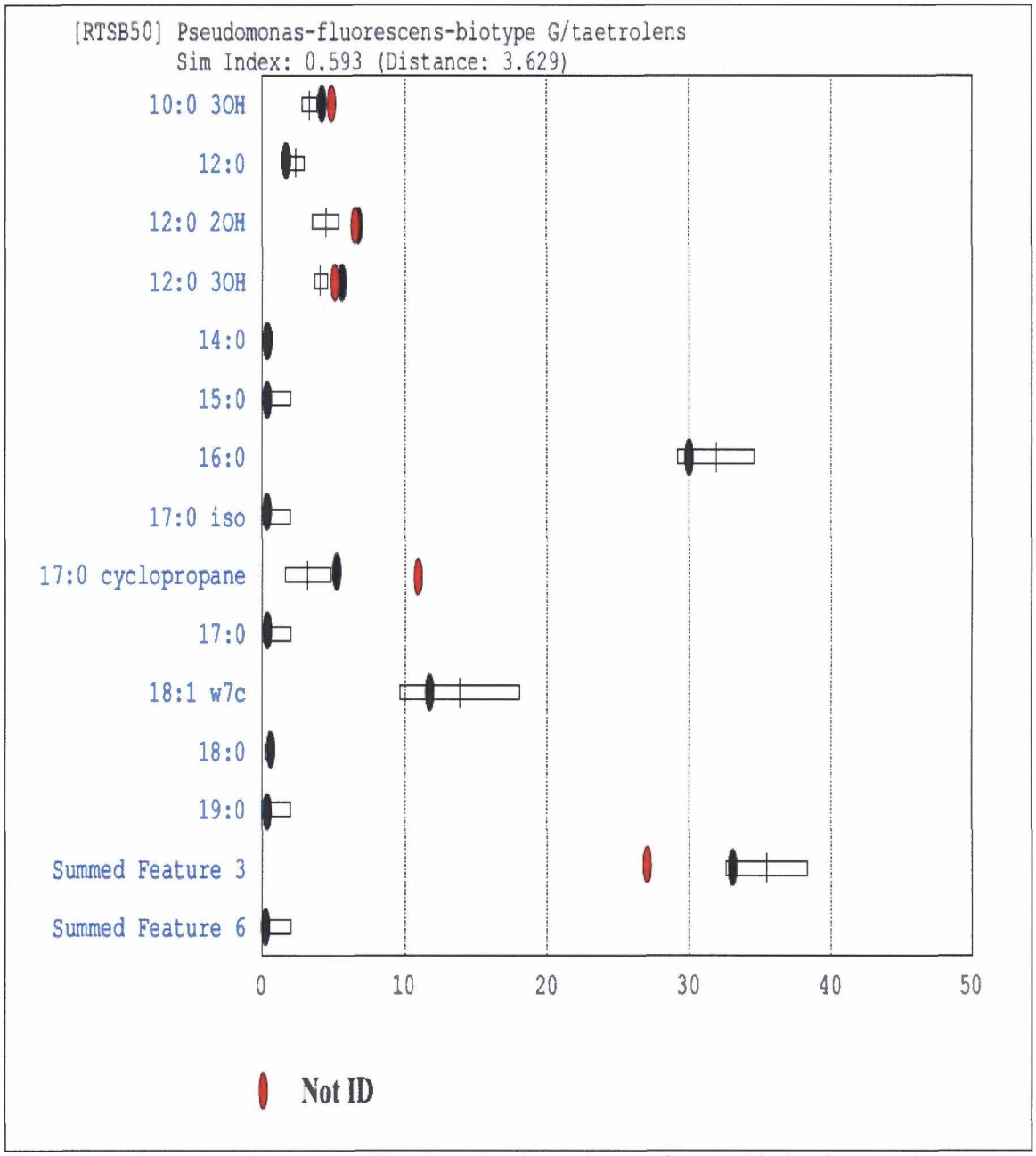

Figure 2.13. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of one replicate of *Pseudomonas fluorescens* 11A1.

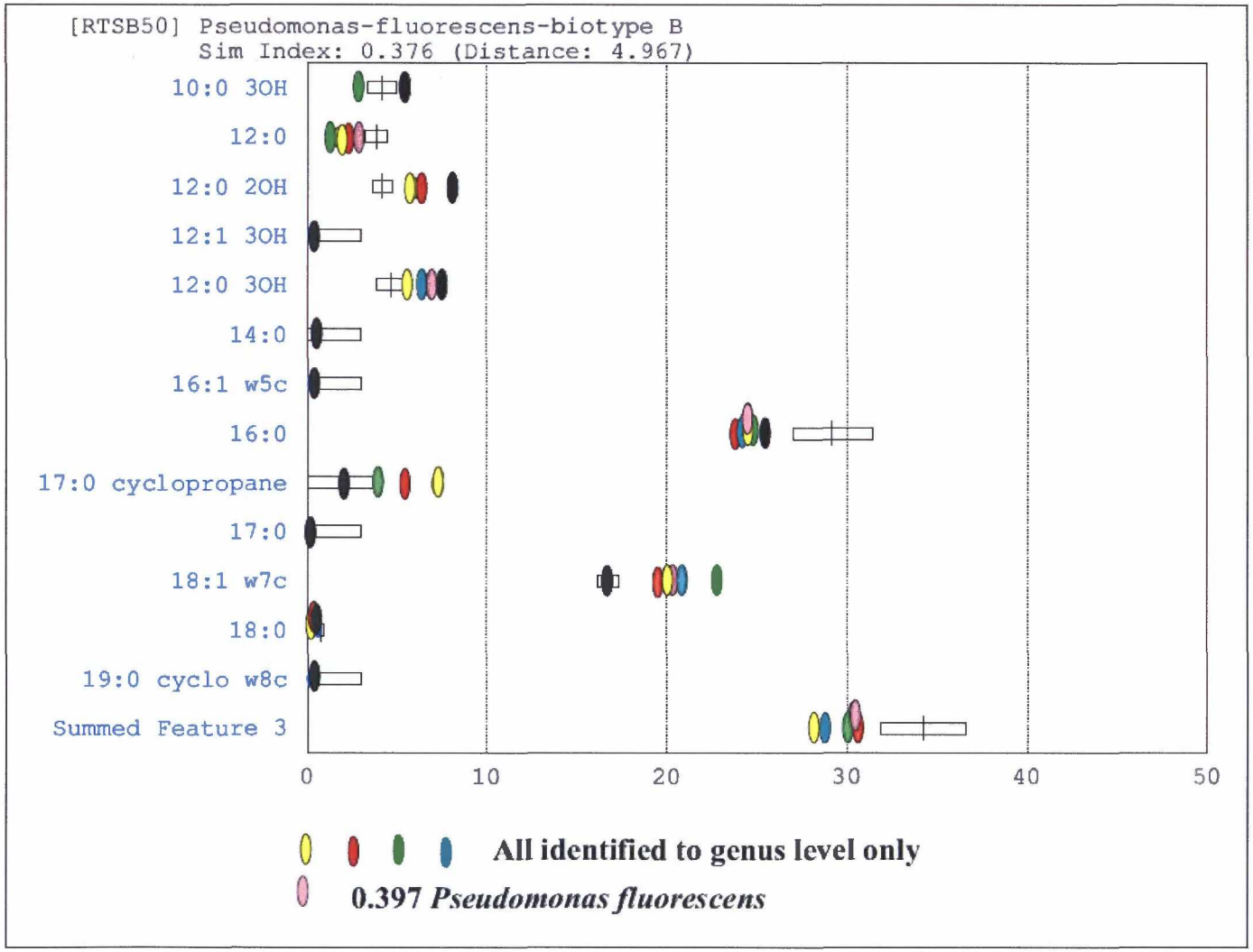

Figure 2.14. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of five replicates of *Pseudomonas fluorescens* 11B3.

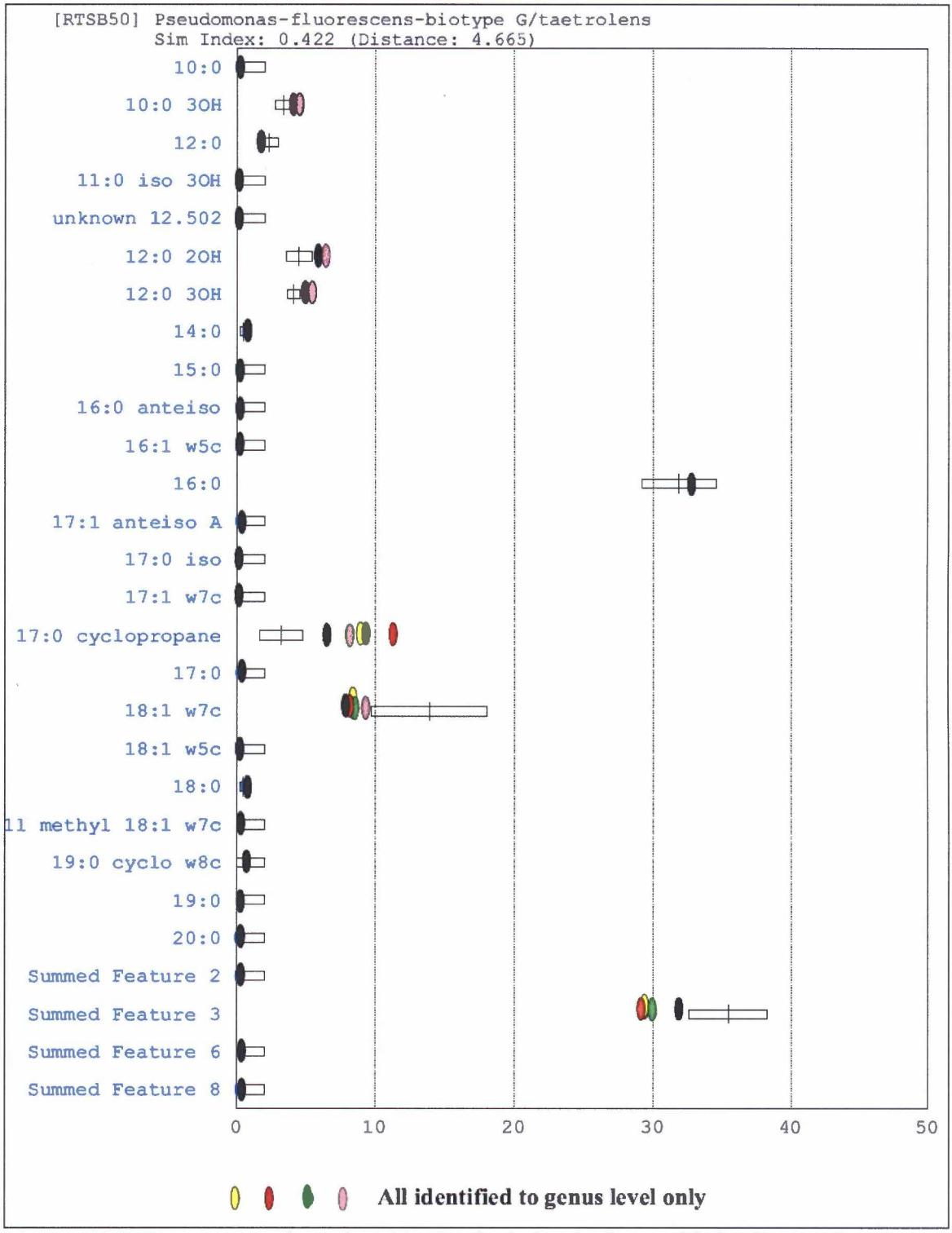

Figure 2.15. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of four replicates of *Pseudomonas fluorescens* 12A2.

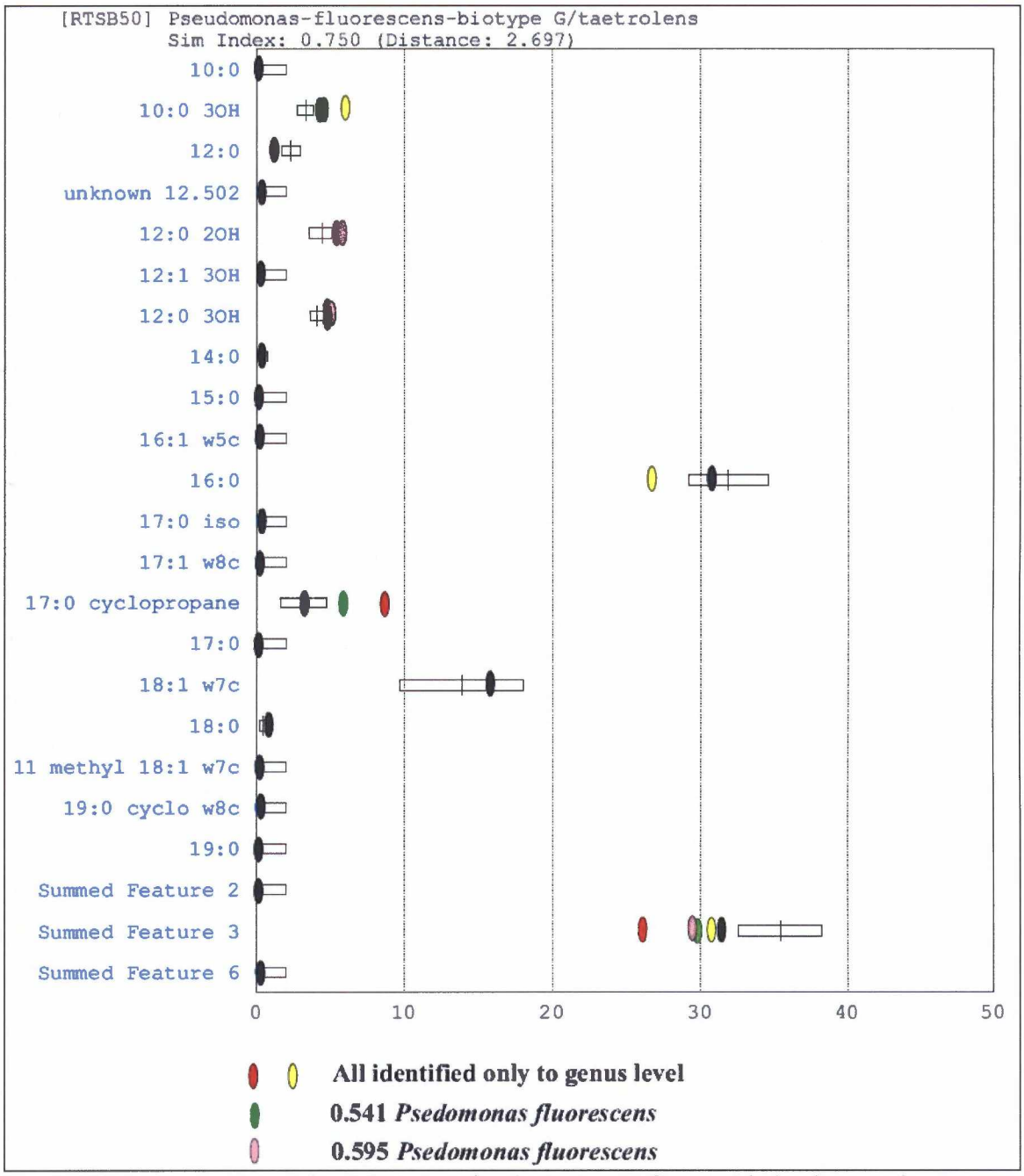

Figure 2.16. Comparison chart showing the deviation in fatty acids leading to the deviation in identification of four replicates of *Pseudomonas fluorescens* ATCC 31419.

Chapter 3: Application of a Rapid Cellular Fatty Acid Based Method for Tracking Bacterial Spoilage in Pink Salmon[1]

3.1. Abstract

Classical taxonomic techniques developed for identification of microorganisms to the species level are time-consuming. The Sherlock Microbial Identification System (Sherlock MIS) software is a rapid bacterial identification system that identifies bacteria through similarity indices when comparing the fatty acid profiles to a database of known bacteria. The objective of this research was to use Sherlock MIS for rapid identification of bacterial species as pink salmon spoils in ice. Fresh pink salmon (*Oncorhynchus gorbuscha*) obtained locally were washed, hand-filleted, packed and stored in ice up to 15 days. In another experiment, gutted pink salmon with ice (IB) and without ice in the belly cavity (B) were stored and microbiological sampling was conducted on gills (G), IB and B on day 0, 4, 6 and 20. Aerobic plate counts (APC) were calculated after colonies had developed at 25° C for 48-72 h. From representative plates, ten colonies were randomly selected, purified and cell wall lipids were extracted, converted to methyl esters and analyzed by gas chromatography using the manufacturer's protocols. The APC for iced pink salmon, skin-on fillets increased from 3 cfu/sq. cm initially to 6.6 log cfu/ sq. cm on day 15. Similarly, the APC of gills and belly cavity irrespective of treatment increased to 8 log cfu/sq. cm or /g in 20 days. Highly mixed bacterial flora in all tissues confirmed the freshness of the day zero fish. *Pseudomonas fluorescens/ putida* formed a major portion of the microflora on the final day of storage while *Shewanella putrefaciens* was in minor proportion, in all tissues. This indicated that spoilage in pink salmon is caused by *P. fluorescens/ putida*. Rapid identification of the pink salmon bacterial species was successful using Sherlock MIS and may be useful in determining the quality of raw salmon stored commercially in ice.

[1]Morey, A., Himelbloom, B.H., Oliveira, A.C.M., 2007. Application of rapid cellular fatty acid based method for tracking bacterial spoilage in pink salmon. Prepared for submission in the International Journal of Food Microbiology.

3.2. Introduction

Bacteriological degradation of the complex biochemical matrix is the major cause of fish spoilage. The total bacterial plate count (TPC) is the common method to determine the microbial quality of fresh fish and the threshold has been set at 10^7 colony-forming units/g (ICMSF, 1978). The duration in which this value can be reached during the post harvest storage depends on a combination of: 1) initial bacterial load: higher initial TPC reach the threshold faster (Jeyasekaran et al., 2005; Hozbor et al., 2006) while cold and clean water fish have lower TPC than warm water species (Gram and Huss, 2000); 2) post-harvest handling measures: treatment with sanitizers (Wempe and Davidson, 1992) and immediate icing (Jeyasekaran et al., 2005) reduce the growth rate; 3) storage conditions: modified atmosphere packaging can delay microbial growth compared to aerobic storage (Lalitha et al., 2005; Fletcher et al., 2002); 4) temperature of storage: lower temperatures slow bacterial growth (Wempe and Davidson, 1992; Ola and Oladipo, 2004; Mejlholm et al., 2005); 5) tissue sampled: slower in increase in TPC in flesh of whole ungutted fish than for fillets (Taliadourou et al., 2003) and 6) composition of bacterial flora: initial mesophilic counts decrease on icing to delay bacterial growth (Jeyasekaran et al., 2005).

The composition of the microflora of fish has been observed by various researchers to be different for geographic locations, species, and storage practices and is dynamic with the storage period and conditions. Although the microflora on fish is diverse, a small group of microorganisms designated as "specific spoilage organisms" (SSO) are able to cause deterioration in the flavor, odor and taste of fish (Gram and Huss, 1996; Gram and Dalgaard, 2002). Bacteria belonging to *Pseudomonas* spp. and *Shewanella putrefaciens* have been identified as SSO in iced fresh fish (Gram and Huss, 1996).

The importance of *Pseudomonas* spp. in seafood spoilage is due to: 1) being isolated from aerobically stored freshwater fish (Garcia-Lopez et al., 2004) as well as tropical fish (Shetty et al., 1992) and temperate water oceanic fish (Himelbloom et al., 1994), 2) ability to produce proteases and/or lipases (Jaeger et al., 1994; Tryfinopoulou et al.,

2002) and 3) produce esters aldehydes, ketones and sulfur compounds except H_2S (Huss, 1995).

Shewanella putrefaciens, can be differentiated from *Pseudomonas* spp. by the ability to produce pink pigments, H_2S and ornithine decarboxylase, and reduce trimethylamine oxide (TMAO) (Stenström and Molin, 1990). *Shewanella putrefaciens* has been demonstrated to have a very high proteolytic activity and produce methyl mercaptans, dimethyl disulfide, dimethyl trisulfide, 3-methyl-1-butanol, H_2S and trimethylamine from reduction of TMAO in fish muscle (Miller et. al., 1973) which leads to the sensory rejection of fish (Ryder et al., 1993). The production of H_2S results from hydrolysis of sulfur-containing amino acids like cysteine and cystine, made available by the fish proteolytic enzymes (Lapin and Koburger, 1974). Also, *S. putrefaciens* can utilize tyrosine and lysine to produce tyramine, and cadaverine and putrescine exhibiting ornithine decarboxylase activity (Baixas-Nogueras et al., 2003b).

The role of SSO is not only restricted to their potential to cause spoilage but also has been extended to their interactions with the other microorganisms present in the fish flora (Chai et al., 1968; Gram 1993, 1994; Baixas-Nogueras, et al., 2003a) which can affect the dynamics of fish spoilage microflora (Gram, 1993).

The identification of the SSO in particular and microorganisms in the spoilage microflora in general can help in modeling the microbial community structure for particular seafood, assessing the interactions within the community, predicting shelflife of a product as well as improvising/devising methods to curb the microbial spoilage (Gram and Dalgaard, 2002). A quick estimation of SSO load can be achieved by the use of selective media like cetrimide fucidin cephaloridine (CFC) agar and iron agar for detection of pseudomonads and *S. putrefaciens* (Taliadourou et al., 2003) but the growth of non-target organisms on CFC agar has been observed (Salvat et al., 1997). Hence, it would be advisable to identify the microorganisms to the species level.

Identification of these microorganisms by classical techniques is a time consuming, labor intensive and sometimes expensive process (Tothill et al., 2003). A solution to these deterrents is the use of manual and automated rapid microbial identification techniques/systems based on genotypic and phenotypic characters of microbes (Odumeru et al., 1999; O'Hara, 2005).

The Sherlock Microbial Identification System (Sherlock MIS, Microbial ID Inc., Newark, DE) is a fully automated system which uses direct computerized comparison of fatty acid analysis of test strains against the library database of more than 100,000 bacterial fatty acid profile entries to provide an identification (O'Hara, 2005). The Sherlock MIS (also referred as the MIDI system) has been used in various fields of microbiology and a list of references is provided by the manufacturer on their website (http//www.midi-inc.com/media/pdfs/Reference_list.pdf). In the field of food microbiology, MIDI system has been used in poultry microbiology (Hinton et al., 2004; Sarjeant et al., 2005), dairy microbiology (Lin et al., 1998; Sengul, 2006) and fresh-cut produce microbiology (Robbs et al., 1996 a,b). The ability of MIDI to identify food-borne pathogens has been studied by Odumeru et al. (1999). However, the literature on the application of Sherlock MIS in the identification of fish and seafood bacteria is rather limited. Nedoluha and Westhoff (1995, 1997a,b) used Sherlock MIS to identify bacteria associated with aquacultured striped bass while Kim, Eun, Chen, Wei, Clemens and An (2004) and Lee, Kim, Wei, Jun, Eun and An (2005) used the system to identify biogenic amine forming bacteria in canned anchovies.

It has been suggested that bacteria attack the flesh mainly through the gills and the kidney via the vascular system and directly though the skin and peritoneum (Shewan, 1962). Hence, the importance of the SSO and the spoilage microflora composition and the need for their rapid identification, this study was conducted to observe changes in microflora using Sherlock MIS for pink salmon stored in ice. The importance of studying skin microflora relates to: 1) external contamination occurs naturally, 2) sampling is quick and non-destructive and 3) the method is a generally-accepted practice for evaluating the

bacterial quality of fish. The ability of Sherlock MIS to identify bacteria associated with seafood was demonstrated in Chapter 2.

The objectives of the research were to:

1. Estimate the total bacterial load on fish skin, gills, belly cavity during iced storage.
2. Identify the bacteria using the cellular fatty acid-based rapid MIDI method for representative isolates associated with fresh and spoiling pink salmon.
3. Track the changes in bacterial flora composition during ice storage of pink salmon.
4. Analyze the fatty acid composition of bacteria lipids extracted from isolates representative of different niches in pink salmon during iced storage.

3.3. Materials and Methods

3.3.1. Fish

Fresh whole seine caught pink salmon (*Oncorhynchus gorbuscha*) caught by seine boats in the Gulf of Alaska and held in chilled seawater less than 24 h were procured from a seafood processing plant in Kodiak, AK. Only those fish which did not show any physical damage were selected, iced and brought to the pilot plant. Fish were de-iced, washed and maintained under iced condition till further processing. Fish were brought on two separate occasions in the summer of 2005 and each batch was used for different experiments.

3.3.2. Fish processing

The experiments were setup to imitate commercial conditions of processing and handling of fillets.

3.3.2.1. Experiment 1: Iced Fillet Storage

Within one hour, the first batch of fish (n = 3) were deheaded, gutted, trimmed to remove the fins, washed and kept ready for further processing. The dressed fish were hand filleted into boneless, skin-on fillets and washed with chilled tap water. Care was taken to remove the blood stains and slime. These washed fillets (n = 6) were placed individually in separate plastic bags. In order to prevent the melting ice water from contact with the fillets, each plastic bag was closed using a rubber band. The bags containing fillets were placed in a tote containing ice. A layer of ice was placed at the bottom of the tote and care was taken that the bags were placed away from the sides and the base of the tote. Finally, the bags were covered with a layer of ice and the tote was closed with a loose fitting lid. The tote was kept in an insulated room maintained at $5 \pm 1^{\circ}C$. The melting ice was replenished every 48 h to 14 days. The average temperature of the fish fillets throughout the study was maintained at $2 \pm 1^{\circ}C$.

3.3.2.2. Experiment 2: Gutted Fish Storage in Ice

Another batch of pink salmon (n = 8) was immediately degutted. The bellies of degutted fish were rinsed thoroughly with tap water to remove any trace of gut and blood. These degutted fish were divided into two treatments and each treatment was stored into different totes. Flake ice was used throughout this experiment. The fish from one batch (n = 4) were iced with their bellies filled with flaked ice (IB), the other batch (n = 4) of fish was ice stored without ice in their bellies (B). Icing was carried out in this manner: a layer of ice at the bottom, then the fish were placed in belly down position without touching each other, the space between the fish was filled with ice. Belly down position allowed the melt water from the bellies to wash away rather than getting collected in the belly cavity in belly-up storage. Each layer of fish was completely covered with flake ice. The depth of fish and ice did not exceed 45 cm in order to minimize the pressure on the fish on the bottom layer. The totes were kept in the insulated temperature ($5 \pm 1^{\circ}C$)

controlled room throughout the study period. The melting ice was replenished every 24 h to 19 days.

3.3.2.3. Sampling strategy

In experiment 1, a random sample was drawn every three days from day 0 to day15. One bag containing fillet was used during each sampling day and was later discarded. In the experiment 2, one fish was take from each treatment (IB and B) sampling was carried out on day 0, 4, 6 and 20. Gill samples were taken from the fish stored without ice in their belly (B).

3.3.2.4. Aerobic plate count (APC)

On each sampling day, the skin, belly and iced belly were separately swabbed for estimation of aerobic plate count using sterile template (area of 10 sq. cm) (Tretsven, 1963). Gills were aseptically cut and 25 g of the sample was macerated into 225 mL of deionized water. Serial dilutions of the sample were made in 0.1% (w/v) of peptone water (Crapo et al., 2004). A 0.1 mL aliquot of each serial dilution was pipetted, spread - plated, in duplicate, on plate count agar (PCA; Difco Lab, Detroit, MI) supplemented with 0.5% w/v NaCl. These plates were incubated at 25°C for 48-72 h for estimation of APC (Himelbloom et al., 1991). After the incubation period, the plates with 30-300 colonies were selected for counting (Harrigan, 1998a). Total number of colonies counted from duplicate plates were averaged and used to calculate colony forming units per sq. cm (as $\log_{10}$ cfu/sq. cm) for skin, belly and iced belly and $\log_{10}$ cfu/g for gills.

3.3.2.5. Characterization of microorganisms

From representative agar plates at each sampling period, ten colonies were randomly selected and labeled according to the number of that particular colony on the APC plate,

day of sampling and tissue, e.g., 3/0/SS2 means colony # 3 on day 0 sampling of salmon skin from APC plate number 2. These ten colonies were subjected to the KOH reaction (Powers, 1995), catalase activity (Harrigan, 1998b) and oxidase test (Kovacs, 1956). Morphological characteristics (shape and motility) of the selected microorganisms was observed under microscope (Zeiss, West Germany, Montagesaez T-UL, 467065-9914) by using the 40X and 100X oil immersion objective.

The selected bacteria were restreaked for purity on PCA. Colonies were harvested and resuspended using 1 ml of brain heart infusion (Difco Lab., Detroit, MI) added to a 4 ml sterile vial containing 1 mL of sterile 50% glycerol (Sigma Chemical Co., St. Louis, MO) and 40 µL dimethylsulfoxide (Sigma). Vials were capped and stored as frozen stocks at -80°C until identification.

3.3.2.6 Identification of bacteria using Sherlock MIS

The bacterial stocks were thawed at room temperature for 15-20 min, a subsample revived on PCA and checked for purity by conducting basic biochemical characterization as in section 3.2.2.5. Pure cultures were then streaked on Trypticase soy broth plus agar (TSBA: 30 g of Trypticase soy broth (BBL, Sparks, MD) and 15 g agar (BBL)) plates and incubated at 28°C for 24h. Fatty acid methyl esters (FAME) were extracted using the procedure of Paisley (2004) as duplicates. The FAME were immediately analyzed on the GC model 6850 (Agilent Technologies, Wilmington, DE) which was coupled with a flame ionization detector (FID). The GC was equipped with an autosampler (Agilent 6850 Automatic sampler G2880A). A split injector was used with a split ratio of 40:1 and 2 µL of the extracted FAME was injected for each analysis. The GC was fitted with Ultra 2 - 5% phenyl methyl silicone fused silica capillary column (25 m x 0.2 mm, Model 19091B-102E, Agilent). Hydrogen gas (99.999% pure) was used as carrier gas at a flow of 1.4 mL/min (velocity = 0.6 m/s) with a pressure of 21.5 psi. The hydrogen gas used was generated in the laboratory using a hydrogen generator (Agilent model 5182-3482). The oven temperature ramped from 170°C to 288 °C at 28 °C/min and then to 310°C at

60°C/min. The total run time was 5.83 min. The FID was supplied with hydrogen (30 mL/min) and air (350 mL/min) and nitrogen (99.999% pure) used as a makeup gas. The nitrogen was supplied by Air Products Inc. (Allentown, PA) while the air was generated in the laboratory using the Zero Air gas generator with compressor (Model ZA035A-ZA180A, Peak Scientific Instruments Ltd., Renfrew, Scotland). The Chemstation enhanced integrator program (G.C. Chemstation rev. A. 10.02, 1757) was used to integrate the chromatogram peaks and electronically transferred to Sherlock MIS for RTSB50 library comparison with the TSBA50 aerobe database and report generation. The GC conditions were monitored strictly by the Sherlock MIS software.

The GC was calibrated using a calibration standard (MIDI Part No. 1300-AA) for the Sherlock Rapid Method. The standard contained FAME of straight chain saturated (9:0 to 20:0) and five hydroxyl fatty acids. The calibration standard was run twice before the start of each analysis sequence as well as after every 11^{th} sample injection (Paisley, 2004). During all the calibration mixture analysis, the peak percent named for the standard was above 99% with the root mean square error below 0.0030.

The Sherlock MIS compares the fatty acid profile of the test organism with those in its library (database) and finds the most likely identification/identifications by using multivariate statistical methods. Based on the Euclidean distance between the test organism and its database, a list of most identification/identifications is populated and the software calculates a number called 'Similarity Index' (SMI) for each identification. Strains with SMI > 0.500 with a separation of 0.100 between first and second choice are considered as good library comparisons. Some exceptions to the above rule were observed and these organisms were applied to the following rules:

1) the first rank in the identification list was considered if it was separated from the second rank by >0.1 SMI,

2) if there were more than one identification within 0.1 SMI of the first rank then based on previous experience with the ATCC and FITCCC culture and knowledge of the seafood microbiology, the identities which were not relevant were not considered.

3) for those isolates where ranks within 0.1 SMI were most likely to be present in seafoods were combined together e.g. if first rank of *Psychrobacter* and second *Moraxella* (or vice versa) (both expected in seafoods) were separated by <0.1 SMI then that particular isolate was attributed to *Psychrobacter-Moraxella* complex similarly for *Moraxella-Acinetobacter*, *Psychrobacter-Acinetobacter*, *Pseudomonas fluorescens/putida*, *Acidovorax/Delftia*, *Acidovorax temperans/avenae* and other similar identifications are mentioned in the results.

There was one group of 15 gram-negative oxidase-positive isolates designated as *Psychrobacter/Moraxella* since the identification fell within one of the following category:

1. first rank of *Moraxella catarrhalis* separated from second rank of *Psychrobacter immobilis* by >0.1 SMI in one replicate but the distance was <0.1 in other replicate,
2. only choice of *Moraxella catarrhalis* in the identification list of the first replicate and an additional choice of *Psychrobacter immobilis* within 0.1 SMI range in the second replicate or vice versa,
3. *Moraxella catarrhalis* as the sole choice in both the replicates but with low SMI of <0.3.

3.4. Results

3.4.1. Aerobic plate count (APC)

The average number of bacteria on the skin surface on the day 0 was 3.04 log cfu/sq. cm which increased gradually to 6.6 log cfu/sq. cm on day 15 (Figure 3.1). The initial APC of the gills and belly were 4.3 log cfu/g and 2.3 log cfu/sq. cm, respectively which decreased in gills to 3.7 log cfu/g and increased to 3.5 and 4 log cfu/sq. cm in iced belly and belly without ice after day 6 of ice storage (Figure 3.2). Storage to 20 days showed the APC was *ca* 8 log cfu/g or sq. cm in all samples.

As per the sampling design, ten random colonies were selected from each sampling day, totaling the isolates from skin and gills to 60 and 40, respectively. Day 0 sampling of belly cavity yielded only 4 colonies while sampling was not done for day 0. Thus the total number of bacterial colonies for the belly and iced belly were 34 and 30, respectvely.

3.4.2. Identification of microorganisms by Sherlock MIS and their fatty acid profiles

3.4.2.1. Acidovorax and Delftia

Ten gram-negative, catalase- and oxidase-positive, motile rods forming non-pigmented colonies isolated from both experiments were classified in family Comamonadaceae either to the genus *Acidovorax* or *Delftia*.

Two isolates belonging to day 0 salmon skin (4/0/SS2) and belly (1/0/B2) were identified as *A. konjaci* as the first rank (SMI 0.788-0.822) in all replicates (Table 3.1). In the identification list of these two isolates, the second rank of *D. acidovorans* was separated from *A. konjaci* by >0.1 SMI unit. The mean fatty acid profiles of these two replicates is given in Table 3.2. There were ten fatty acids comprising the profile of *A. konjaci* with 16:0, 16:1 ω6c/ω7c and 18:1 ω7c as major fatty acids. The fatty acid profiles of these two organisms were similar to those of *A. konjaci* (Gardan et al., 2000, 2003) with an exception for the absence of 17:0 cyclopropane and a higher percentage (15.6 to 17.8%) of 18:1 ω7c in the later study.

For four other isolates, day 0 and day 3 salmon skin and two from day 6 gills, the first two rankings of *A. konjaci* and *D. acidovorans* were <0.1 SMI unit in both replicates (Table 3.3.). The replicate analysis of day 0 salmon skin showed the first rank as *D. acidovorans* and the second rank as *Variovorax paradoxus*. The mean fatty acid profiles of these isolates is shown in Table 3.2 and were similar to the two strains of *A. konjaci*.

Among the remaining isolates; one each was from day 6 gills and belly and identified as *A. avenae citrulli* in both replicates with an SMI range of 0.450-0.609 (Table 3.4). The other two isolates were from day 0 gills. One was identified as *A. temperans* as first rank and the other as *A. avenae citrulli*. The fatty acid profiles (Table 3.2) for the two day 0 gill isolates (7/0/G2 and 5/0/G1) identified as *A. avenae citrulii* or *A. temperans* conformed to the fatty acid profiles of *A. avenae citrulii* (Gardan et al., 2000, 2003). The day 6 isolates although identified only as *A. avenae citrulii* showed an absence of 15:1 ω6c and a higher content of 14:0 (~4.5%) compared to fatty acid profiles for two other *Acidovorax* spp. (Gardan et al., 2000, 2003).

3.4.2.2. Aeromonas

Five isolates from day 4 gills and day 6 belly and gills were identified as belonging to genus *Aeromonas* (Table 3.5). Four isolates were identified as *A. ichthiosmia* or *A. hydrophila* (SMI of 0.735-0.825) among which *A. salmonicida masoucida* could be the first rank or <0.1 SMI unit of the first rank during replicate analysis. The remaining isolate from day 4 gills was identified as *A. hydrophila* but the SMI was extremely low (<0.100) in two separate replications. The fifth isolate from day 6 gills was identified as *A. veronii* as the first rank in the identification list in both replicates. However, the first replicate was identified with a low SMI (0.204) as *A. veronii* while the replicate showed a high SMI (0.764) with the same identification.

The mean fatty acid profiles of all fatty acids >1% for *A. ichthiosmia/hydrophila* and *A. veronii* are shown in Table 3.6. The major fatty acids comprised 16:0, 18:1 ω7c and SF 3 (16:1 ω6c and/or 16:1 ω7c). The fatty acid profiles were similar for all the isolates except a day 4 gill isolate, which showed the presence of 10:0 3-OH, 12:0 3-OH, 15:0, 17:1 ω8c and 17:0.

3.4.2.3. Brevundimonas

Five of six isolates, from day 0, 3, and 6 salmon skin, day 0 belly and day 4 gills were identified as *Brevundimonas vesicularis* as the first rank (SMI range of 0.745-0.884). The sixth isolate (day 0 belly) was identified as *B. diminuta* in both replicates without a second rank listed (Table 3.7). The mean fatty acid profiles of *B. vesicularis* and *B. diminuta* are shown in Table 3.8 and the major fatty acids were 18:1 ω7c and 16:0.

3.4.2.4. Flavobacterium and Chryseobacterium

Three isolates from day 0, 3 and 12 salmon skin were identified as *F. johnsoniae* (SMI of 0.589-0.613) although one isolate had a low SMI (mean of 0.16) (Table 3.9). The day 6 gill isolate was identified as *C. meningosepticum* or as *Pedobacter heparinus* and the iced belly isolate was identified as *C. indologenes* (Table 3.9). The mean fatty acid profiles of the five *Flavobacterium* and *Chryseobacterium* spp. are shown in Table 3.10. The major fatty acids were 15:1 iso G (the letter 'G' after the fatty acid indicates that position of the double bond is not known), 17:0 iso 3-OH, 16:1 ω6c/16:1 ω7c and 10-methyl-16:0/17:1 iso ω9c. The major fatty acids of *F. johnsoniae* were 15:0 iso, SF 3 (i.e. 16:1 ω6c/ω7c), 17:0 iso 3-OH, 15:0 iso 3-OH and 16:0 3-OH. *C. indologenes* had a higher content of 15:0 iso, 17:0 iso 3-OH and SF 4 (i.e., 10-methyl 16:0/17:1 iso ω9c) compared to *F. johnsoniae*. Similarly, *C. meningosepticum* (currently *Elizabethkingia meningoseptica*) had the highest percentage of 15:0 iso and SF 3 (16:1 ω6c/ω7c) and lowest percentage of SF 4. The reason for the high standard deviation in 15:1 iso G of *F. johnsoniae* was due to the varying percentage (1.74%, 1.50% and 5.73% respectively for each isolate) of this fatty acid.

3.4.2.5. Psychrobacter, Moraxella and Acinetobacter

Sixty one isolates were identified as belonging to a complex composed of the closed related genera of *Psychrobacter*, *Moraxella* and *Acinetobacter*. *Psychrobacter immobilis* was the first rank identified for salmon skin isolates (17 of 36), gills (13) and iced bellies (6). The SMI ranged from 0.061 to 0.918 for 34 isolates (Table 3.11).

A total of 55 fatty acids were identified in the 36 isolates identified as *P. immobilis*. Figure 3.3 depicts the fatty acids which were either more than 1% or more than 85% of occurrence among all isolates. It can be observed that the prominent fatty acids (100% occurrence) comprised of *P. immobilis* were 18:1 ω9c, 17:1 ω8c and SF 3 followed by 10:0, 12:0 3-OH and 18:0. It should be noted that 62% and 49% of the *P. immobilis* strains possessed 18:3 ω6, 9,12c (range 0-3.86%) and 20:4 ω6, 9, 12, 15c (range 0-0.53%) respectively.

As mentioned in the material and methods section, 15 isolates were designated as *Psychrobacter/Moraxella*. Only one isolate from day 20 gills was identified as *M. catarrhalis* (*Branhamella catarrhalis*) because it was the sole bacterium in the identification list.

The basic biochemical characterization of seven oxidase-negative coccobacilli corresponded well with these isolates being identified as *Acinetobacter*. Four isolates were identified as *A. calcoaceticus* as a top rank (Table 3.12). One isolate from day 6 belly was also identified as *A. calcoaceticus* but had a very low SMI (<0.05) for replicates. Two day 6 belly isolates were identified as *A. calcoaceticus* with the replicate identified as *A. johnsonii* (Table 3.12).

The fatty acid profiles of the isolates identified as *P. immobilis*, *Psychrobacter/Moraxella*, *Acinetobacter* and *Psychrobacter/Moraxella/Acinetobacter* are shown in Figure 3.3, 3.4, 3.5 and 3.6, respectively. The common major fatty acids for each were 18:1 ω9c and SF 3. The fatty acid profile of 15 isolates belonging to

Psychrobacter/Moraxella group shows the fatty acids which were present in all isolates (i.e., 85% to 100% occurrence) and those fatty acids with maximum percentage of more than 1%. The major fatty acids were 18:1 ω9c, SF 3, 17:1 ω8c, 12:0 3-OH, 16:0 and 10:0. There were 7 isolates identified as *Acinetobacter* spp. and the average fatty acid profile of these isolates shows the major fatty acids with were 18:1 ω9c, SF 3, 16:0 followed by 12:0 and 12:0 3-OH. There were three isolates identified with SMI < 0.1. which were identified as belonging to either *Acinetobacter/Psychrobacter/Moraxella.* The major fatty acids of this group were SF 3, 18:1 ω9c and 17:1 ω8c. The fatty acid, 17:1 ω8c, was absent in the isolates identified as *Acinetobacter* spp.

3.4.2.6. Pseudomonas fluorescens/putida

Forty-three isolates from gills, bellies and skin on various days of iced fish storage were classified as genus *Pseudomonas*. Forty-two isolates were identified as *P. fluorescens/putida* SMI of above 0.5 for replicates. One day 0 salmon skin isolate was identified as *P. stutzeri* as the first rank in the replicates. This isolate had a similar fatty acid profile as the forty-two *P. fluorescens/putida* isolates but could be separated from the rest by the presence of 9.7% 12:0 compared to 1.2 – 4.7% and absence of 12:0 2-OH compared to 3.9 – 7.2%. Major fatty acids for *P. fluorescens/putida* were SF3, 16:0 and 18:1 ω7c (Figure 3.7).

3.4.2.7. Shewanella putrefaciens

Seven isolates were identified as *S. putrefaciens* (SMI range of 0.215-0.780) for replicates. The fatty acid profile of *S. putrefaciens* is given in Table 3.16 and the major fatty acids were SF 3, 17:1 ω8c, and 15:0 iso.

3.4.2.8. Miscellaneous organisms

Four isolates (day 0 salmon skin, day 4 belly cavity and day 6 iced bellies) were identified as *Stenotrophomonas maltophilia* (Table 3.13). Seven isolates (four from day 4 and three from day 6 iced bellies) and two isolates from day 0 salmon skin were identified as *Staphylococcus epidermidis* (Table 3.14). Two isolates one from day 0 belly and day 3 salmon skin were identified as *Staphylococcus xylosus/cohnii* (Table 3.14). Two isolates, day 0 and 6 gills, were identified as *Pseudoalteromonas nigrifaciens* and one isolate from day 6 belly cavity was identified as *Xanthobacter flavus* (Table 3.15). One isolate from day 6 salmon skin was identified as *Microbacterium saperdae/Kocuria kristinae* (Table 3.15). Fatty acid profile of the miscellaneous organisms is given in Table 3.16.

3.4.3. Changes in microflora of salmon skin during ice storage

The ice storage of pink salmon skin-on fillets was conducted for 15 days with sampling every three days. Of 60 isolates, 56 were gram-negative and only four were gram-positive. The microflora changes on salmon skin during iced storage are shown in Figures 3.8-3.10. The day 0 microflora was composed of seven bacterial genera, while on day 3, half of the isolates were *Psychrobacter immobilis*. At day 6, the microflora of salmon skin comprised the SSO *Pseudomonas fluorescens/putida* (Figure 3.9). The other SSO, *Shewanella putrefaciens*, was not detected until day 12 and composed only 10% of the microflora. On day 15, *P. fluorescens/putida* comprised 60% and *S. putrefaciens* 20% of the microflora.

3.4.4. Changes in microflora of gills, belly cavity and iced belly during ice storage

On days 0, 4, 6 and 20, ten colonies were isolated from the APC plates except for the day 0 belly since the APC was very low. From 104 isolates, 97 were gram-negative and three isolates were gram-positive. The proportion of each bacterial isolate in the microflora is

shown in Figures 3.11-3.14. Initially, the microflora of gills comprised of *Psychrobacter/Moraxella* (30%), *Acidovorax* (20%) while *P. nigrifaciens* and *S. putrefaciens* comprised 10% each. On day 4, the microflora of bellies and gills was qualitatively and quantitatively different (Figure 3.12). Belly cavity without ice was primarily had *A. calcoaceticus/ johnsonii* and *P. fluorescens/ putida* while iced belly was primarily possessed *P. immobilis* and *S. epidermidis*. Gill microflora consisted of the *P. immobilis*, *Psychrobacter/ Moraxella* complex. On day 6 of iced storage, the microflora of each sampled fish tissue remained diverse. The gills consisted of *Aeromonas* and *P. immobilis* while the belly cavity contained *Acinetobacter* and in iced belly *S. epidermidis* was predominant. A wide variation in the microflora from day 0 to day 6 in all niches (gills, bellies and skin) occurred, but the microflora on the final day of iced storage for the three fish sites were comprised of *P. fluorescens/putida* (40-80%) and *S. putrefaciens* (10%).

3.5. Discussion

Fish caught in cold clean waters generally have a lower APC than the ones from warmer waters (Shewan, 1977). In the present study, pink salmon caught in the cold Alaskan ocean waters had a similar bacterial flora to the genera found on other fish sampled in a previous study (Himelbloom et al., 1991). Lower initial counts on the belly cavity indicate that the intestines, which harbor higher number of bacteria than the ocean water (Cahill, 1990), were intact and did not contaminate the belly cavity. Further reduction in the APC of skin and belly cavity would have been due to the removal of bacteria during the on-board chilled sea-water fish storage (Himelbloom et al., 1994) and subsequent evisceration and thorough washing (Shewan, 1962; Tretsven, 1963) before conducting laboratory studies. Subsequent growth of bacteria on the skin surface, gills and bellies were attributed to the psychrotrophic or psychrotolerant nature of bacteria on temperate water fish which are already adapted to the chill temperatures (Gram and Huss, 2000).

Low APC observed in gills and both the belly treatments during storage might be due to effect of melting ice on the removal of bacteria (Cobb et al., 1976) as well as leaching of nutrients such as flavoring compounds, minerals and water soluble proteins (Ashie et al., 1996). Since the melt ice was replaced every 24 h, the bacterial and nutritional load in the storage system was reduced drastically leading to low bacterial load on fish.

The APC on the skin reached 5.6 log cfu/sq. cm after 15 days of ice storage with a sudden exponential growth between 12 and 15 storage days (Figure 3.1). Although the initial count of belly was approximately similar to the skin, the APC reached approximately 8 log cfu/sq.cm while gills with a higher initial APC count reached approximately 7 log cfu/g, after 20 days of storage (Figure 3.2).

The genus *Acidovorax* (Willems et al., 1990) and *Delftia* (Wen et al., 1999) were previously classified as members of *Acidovorans* rRNA homology group III for *Pseudomonas*. *Acidovorax* was formed initially with clinical isolates, namely *A. delafieldii*, *A. temperans* and *A. facilis* (Willems et al., 1990) and Willems et al., (1992) included the phytopathogenic species of *Pseudomonas*, namely *P. pseudoalcaligenes* subsp. *konjaci*, *P. pseudoalcaligenes* subsp. *citrulli*, *P. avenae*, *P. rubrilineans*, *P. setariae* and *P. cattleyae*. *Delftia* can be isolated from clinical samples as well as fresh water, soil and activated sludge but has not been reported from marine water or infected plants (Wen et al., 1999).

Throughout the literature on seafood spoilage microbiology, researchers use CFC agar or King's B agar for isolating and reporting total *Pseudomonas* spp. counts. King's B agar can support the growth of *Acidovorax* spp. (Gardan et al., 2000) while growth of *Acidovorax* on CFC agar is unknown. *Delftia* spp. has been demonstrated to grow on both King's B and CFC agars (Stampi et al., 1999; Wen et al., 1999). Although these two genera were present on fish, they may be included as *Pseudomonas* spp. unless additional confirmatory tests are conducted. The changes in taxonomy and identification of bacterial isolates belonging to *Acidovorax* and *Delftia* in the current study are comparable to other studies. Nedoluha and Westhoff (1995, 1997a,b) used Sherlock MIS for bacterial

identification from hybrid striped bass and have grouped the organisms belonging to genera *Acidovorax*, *Comamonas* and *Hydrogenophaga* spp. under the family Comamonadaceae without giving specific details about identifications of the isolates. Although present on the salmon skin until day 3 and in very low numbers in gills and bellies plus the lack of interest among researchers regarding these species (compared to *Pseudomonas* and *S. putrefaciens*) indicates the unimportance of *Acidovorax* and *Delftia* in fish spoilage. This notion could be confirmed by conducting further studies for determining spoilage capabilities.

In the present study, *Aeromonas* was isolated from fish gills and belly stored in melting ice. There is a possibility that the bacteria dislodged from the gills or from skin by melt water might become established in the belly cavity. A similar hypothesis was made by Nedoluha and Westhoff (1997a, b) who found that the microflora of gills, skin and the fish growing water were related in a recirculating aquaculture system. Iced belly did not show any *Aeromonas* population as the ice in the belly, which did not melt, might have prevented the flow of bacterial load with the melt water.

These strains were gram-negative, catalase- and oxidase-positive, motile rods and their identification as *A. hydrophila* and *A. veronii*, both motile aeromonads (Martin-Carnahan and Joseph, 2005) can be accepted. Based on 16S rRNA studies, *A. ichthiosmia* is identical to *A. veronii* biovar *sobria* (Collins et al., 1993). Hence, the identifications of the isolates as *A. ichthiosmia/hydrophila* indicated that either these bacteria were *A. veronii* biovar *sobria* or *A. hydrophila*. Other taxonomic tests are necessary to differentiate these isolates.

Gram-negative, catalase- and oxidase-positive, motile, short rods producing brownish discoloration (Gilardi, 1978) in TSBA after 24-48 h at 28°C were identified as *B. vesicularis* and *B. diminuta* (Vancanneyt et al. 2005). *Pseudomonas diminuta* and *P. vesicularis* belonging to rRNA group IV were delineated from the genus *Pseudomonas* and reclassified into a new genus *Brevundimonas* based on genotypic and phenotypic studies (Segers et al., 1994). Although bacteria belonging to this genus have been isolated

from clinical specimens (Vancanneyt et al., 2005), *B. vesicularis* has been isolated from modified atmosphere packaged sole fillets (Franzetti et al., 2001), spoiled shrimps (Fu et al., 2002), farmed turbot stored in ice (Rodriguez et al., 2003), gills of cultured blue fin tuna from the Adriatic Sea (Kapetanovic et al., 2006) while *B. diminuta* was isolated from iced Mediterranean hake (Baixas-Nogueras et al., 2003a). *Brevundimonas vesicularis* and *B. diminuta* possess proteolytic activity (Rodriguez et al., 2003) and amino acid decarboxylase activity (Baixas-Nogueras et al., 2003b). *Brevundimonas vesicularis* is identified as an active spoiler due to the ability to produce putrefactive foul odors in shrimp (Fu et al., 2002). Based on the literature and the identifications by Sherlock MIS, *B. vesicularis* and *B. diminuta* were part of the pink salmon spoilage microflora but their role in spoilage needs to be investigated.

The mean fatty acid profiles of *B. vesicularis* differed from *B. diminuta* in terms of higher 16:1 ω6c/ω7c and 18:1 ω6c/ω7c and lower 16:0 and 19:0 cyclopropane (Table 3.8). Similar differences within the fatty acid profiles of *B. vesicularis* and *B. diminuta* were 16:1 ω7c ($7.3 \pm 1.9\%$ and $2.2 \pm 0.7\%$), 18:1 ω7c ($59.0 \pm 4.0\%$ and $45.3 \pm 6.5\%$), 16:0 ($21.2 \pm 2.6\%$ and $30.4 \pm 2.2\%$), 19:0 cyclopropane (<1.0% and $10.1 \pm 3.7\%$) as observed by Segers et al. (1994).

The genus *Flavobacterium* is comprised of nonmotile, gram-negative, catalase- and oxidase-positive rods (Holmes et al., 1984) and has been emended into various genera including *Chryseobacterium* (Vandamme et al., 1994) and *Myroides* (Vancanneyt et al., 1996a). *Chryseobacterium* was further emended by Kim et al. (2005) into a new genus *Elizabethkingia* and renamed *C. meningosepticum* as *E. meningoseptica*. Most seafood microbiology researchers have observed the presence of these bacteria and are reported only as *Flavobacterium* spp. Hence, the exact speciation of *Flavobacterium* spp. associated with seafood is limited.

Flavobacteria are associated with fresh fish skin, gills, intestinal tracts and eggs of fresh and marine, temperate and tropical waters (Cahill, 1990; Austin, 2006) as well as fish spoilage microflora with counts inversely proportional to storage period (Himelbloom et

al., 1994; Magnussson and Martinsdottir, 1995; Crapo and Himelbloom, 1999; Gennari et al., 1999; Ola and Oladipo, 2004). Similarly in the present study, bacteria identified as *Flavobacterium* and *Chryseobacterium* were low in number but were consistent on the salmon skin until day 12, whereas only one isolate was obtained from iced belly and none from the gills or non-iced belly.

Based on the identification of the day 0 and 3 salmon skin isolates as *F. johnsoniae*, which was the only species in the identification list, this particular identification was accepted. The day 6 and 12 isolates had low SMI but the closeness of first and second ranks in the bacterial identification list as *Flavobacterium* and *Chryseobacterium* confirmed the taxonomy of these isolates based on Sherlock MIS. The isolate from iced belly had a very high SMI for the confirmed identity of *C. indologenes*. Osterhout et al. (1991) evaluated the Sherlock MIS system for its identification capability of the clinical isolates. They found that Sherlock MIS could identify 17 of 20 strains of *F. indologenes* (*C. indologenes*) and only 3 of 14 strains of *F. meningosepticum* (*C. meningosepticum*) with an SMI >0.5 as the top rank. Although Nedoluha and Westhoff (1995, 1997a,b) used the Sherlock MIS system for the identification of bacterial isolates from aquacultured striped bass, they did not report identifying isolates to the species level; instead, they grouped these as *Flavobacterium/Cytophaga* (Nedoluha and Westhoff, 1995) and as *Flavobacterium/Cytophaga/Chryseobacterium/Sphingomonas* (Nedoluha and Westhoff 1997a, b).

The spoilage potential of 132 strains of *Flavobacterium* was studied in which 70% grew at 2°C, 50% were proteolytic and 16% reduced TMAO (Castell and Mapplebeck, 1952). They concluded *Flavobacterium* spp. are not as important as other fish spoilage bacteria. Other spoilage studies of *Flavobacterium* spp. using inoculated packs (Lerke et al., 1965; Gennari et al., 1999) and substrate utilization pattern of *Myroides* (Gonzalez et al., 2000) lead to the conclusion these bacteria are not active fish SSO. In contrast, *Flavobacterium* spp. isolated from spoiling mahimahi was shown to possess the urocanic acid production from histidine (Baranowski, 1985), cold-adapted protease production by *F. balustinum*

(Morita et al., 1998) and amino acid-decarboxylase activity of *C. indologenes* isolated from iced Mediterranean hake (Baixas-Nogueras et al., 2003b). The role of non-proteolytic *Flavobacterium* in the spoilage microflora can be the conversion of tryptophan, released by proteolytic bacterial species like *Pseudomonas*, into indole (Ashie et al., 1996).

The fatty acid profile of *F. johnsoniae* in the present study was similar to that obtained for *F. johnsoniae* (Bernardet et al., 1996) except for higher percentage of 15:0, 15:1 iso G, 16:0 iso 3-OH and absence of 15:0 3-OH in their study. *Chryseobacterium meningosepticum* identified in the present study had a similar qualitative fatty acid profile with quantitative differences with *C. meningosepticum* (*E. meningoseptica*; Kim et al., 2005). Differences occurred in the presence of 1.9% for SF 4 (10-methyl 16:0/ 17:1 iso ω9c) and 24.3% for SF 3 (16:1 ω6c/ω7c) compared to 7.8% for 17:1 iso ω9c and 19.6% for 15:0 iso 2-OH and/or 16:1 ω7c/t (Kim et al., 2005).

The gram-negative, non-motile coccobacilli belonging previously to family Neisseriaceae (Bovre, 1984) are now classified under Gamma-Proteobacteriaceae, order Pseudomonadales and family Moraxellaceae (Juni and Bovre, 2005). This family is divided into oxidase-positive genera *Moraxella* and *Psychrobacter* and oxidase-negative *Acinetobacter* (Juni and Bovre, 2005).

The identifications of all bacterial isolates having cell morphology (coccobacillus), colony morphology (non-pigmented round colonies) and basic biochemical characterization (gram-negative, catalase-positive oxidase-positive) corresponded with *Psychrobacter*, *Moraxella* and (oxidase-negative) *Acinetobacter*. Hence, it can be confirmed that all the identifications were correct to the genus level. Ambiguity does exist at the species level especially with the *Psychrobacter-Moraxella* and *Acinetobacter-Moraxella* groups due to a very low SMI and the closeness of the identifications (<0.1 SMI unit of the first rank). The Sherlock MIS, version 4.5 with RTSB 50 library, consists of only one entry under genus *Psychrobacter* (*P. immobilis*) while the other novel species are absent. A major drawback of using any commercial system is the dependence on the

manufacturer's database and the delay in updating can lead to a faulty or unclear identification.

Bacteria belonging to the *Psychrobacter-Moraxella-Acinetobacter* (PMA) group have been frequently isolated from fish during the spoilage process (Cahill, 1990; Gonzalez et al., 2000). The decreasing but consistent occurrence of *P. immobilis* and the *Psychrobacter-Moraxella* group was observed throughout the storage in all the tissues while *Acinetobacter* is not found on day 15 of spoiled herring skin and day 20 for gills, belly and iced belly of freshwater fish (Crapo and Himelbloom, 1999; Gonzalez et al., 2000). The reduction in the percentage of all Moraxellaceae can be due to competitive exclusion of these non-motile organisms by motile *Pseudomonas* spp. during the later part of storage (McMeekin, 1977).

Hozbor et al. (2006) isolated *Moraxella-Acinetobacter* from spoiling ice-stored sea salmon and found that these bacteria were non-hydrogen sulfide producers but produced weak fishy or ammoniacal odors in fish juice. Gonzalez et al. (2000) studied the diversity of family Moraxellaceae in freshwater pike and trout and found *P. immobilis*, *P. phenylpyruvicus* and *P. urativorans*, *Moraxella* spp. and *A. johnsonii*, *A. lwoffii* and *A. calcoaceticus*. They observed a close phenotypic association for the *PMA* group based on carbon substrate utilization and DNA relatedness and found 42% of the isolates identified as *P. immobilis* were highly lipolytic (Gonzalez et al., 2000). Enzymatic activity on these lipid-based substrates can lead to the formation of free fatty acids during spoilage (Tarr, 1954; Gennari et al., 1999). Van Spreekens and Toepoel (1981) observed that only 32% of the *Moraxella* spp. isolated from spoiled shrimp produced TMA while none could produce hypoxanthine but a variety of odors could be produced. The common odors on fish and shrimp were musty, fruity and aromatic while odors like fresh fish, sour, boiled milk, fishy, hay, rubbery were typical to fish and cheesy, neutral, ammonical and rotten cabbage odors were typical to shrimp (Van Spreekens and Toepoel, 1981). Chen and Levin (1974) reported the production of phenethyl alcohol from phenylalanine and ethanol by *Achromobacter* (gram negative coccobacilli) and *M. nonliquefaciens* ATCC

17955 while other *Moraxella* and *Acinetobacter* isolates from fish were negative. Since the organisms previously classified as *Achromobacter* have been reclassified into *Moraxella* then *Psychrobacter* (Gennari et al., 1999), the *Achromobacter* are likely *Moraxella* or *Psychrobacter*.

Acinetobacter can be easily identified from oxidase-positive genera in the *PMA* group. *Acinetobacter* isolated from meat has shown proteolytic activity and can degrade the skin collagen facilitating entry to other bacteria (Gill and Penney, 1977). *Acinetobacter calcoaceticus* is also able to use taurine as a single nitrogen source and releases sulfoacetaldehyde (Weinitschke et al., 2005). Presence of these bacteria throughout storage irrespective of sampling regimen could indicate their importance in pink salmon spoilage but these bacteria contribute little to fish spoilage (Gram et al., 1987; Himelbloom et al., 1994).

The list of identifications of various isolates identified as *P. fluorescens/putida* consisted of names like *P. chlororaphis/aureofaciens*, *P. vancouverensis* and *P. syringae* within the 0.1 SMI range of the first choice. Using *P. syringae* as the identification of the isolates from fish can be ruled out as this is a phytopathogenic species (Palleroni, 2005). As for *P. chlororaphis/aureofaciens* (both fluorescent pseudomonads), it has been repeatedly observed that these species have grouped in the same hydroxyl fatty acid cluster (Oyaizu and Komagata, 1983) and same whole-cell fatty acid subcluster (Vancanneyt et al., 1996b). Moreover based in the biochemical characteristics, *P. chlororaphis/aureofaciens* have also been shown to be in the same cluster as *P. fluorescens* and *P. putida* (Arnaut-Rollier et al., 1999). *P. chlororaphis* has been reported to be isolated from various sources like soil, plants, caterpillar (Haynes and Rhodes, 1962), dead larvae of cockchafer (a large European beetle) and other sources including water (Palleroni, 2005). We did not find any published literature on the isolation and identification of *P. chlororaphis* and *P. aureofaciens* from spoiling seafood.

The occurrence of *P. fluorescens* and *P. putida* is widely cited in seafood microbiology research papers. In the identification of the known cultures of *P. fluorescens* and *P.*

putida (see Chapter 2) a similar occurrence of *P. chlororaphis/aureofaciens* and *P. syringae* in the possible list of identifications was observed. *Pseudomonas fluorescens* and *P. putida* were not observed on fish skin until day 6. While these bacteria were present in day 4 bellies, a decrease was observed on day 6 which might be due to the removal of bacteria by melting ice during storage (Chen and Chai, 1982). The occurrence of *P. fluorescens* and *P. putida* increased as the ice storage reached the final day of storage (Taliadourou et al., 2003; Massa et al., 2005) with a corresponding decrease in the variety of bacterial species present during storage (Himelbloom et al., 1994). About 60% of the microflora consisted of pseudomonads on the final day of ice storage. The dominance of these organisms during the final day of storage can be attributed to the ability of *Pseudomonas* to grow rapidly at chill temperature (Massa et al., 2005) and siderophore production resulting in suppressed growth of other bacteria (Gram, 1993).

The importance of *Pseudomonas* spp. as SSO can be attributed to the ability of psychrotrophic *Pseudomonas* strains to utilize amino acids at temperatures as low as 0.4°C and hence cause spoilage in fish held on ice (Zachariah and Liston, 1973). Psychrotrophic *Pseudomonas* are capable of producing proteolytic enzymes at 10°C and some strains are able to maintain the enzyme activity at 0°C (Hoshino et al., 1997). Proteolysis of fish muscle by *Pseudomonas* results in rotten and putrid odors in fish (Castell and Greenough, 1957) while the sweet and fruity odors (Olafsdottir et al., 2006) and oniony and potato-like spoilage odors can be attributed to the non-proteolytic non-pigmented *Pseudomonas* spp. (Castell and Greenough, 1957). Specifically, *P. fragi* has been identified as the causative bacteria for oniony, fruity odors (Castell and Greenough, 1959). Miller et al. (1973) reported the production of methyl mercaptans and dimethyl disulfide as major sulfur compounds by *P. fluorescens* isolated from ocean perch. Also, *P. fluorescens* is capable of producing purine nucleoside phosphorylase to convert inosine to hypoxanthine (Surette et al., 1990) and produce lipases (Shaw and Latty, 1982; Roy, 1981). *Pseudomonas putida* is recognized as a less important spoilage bacterium due to a lack of proteolytic activity (Gennari and Dragotto, 1992) but isolates from Mediterranean hake produced putrescine (Baixas-Nogueras et al., 2003b).

Based on all the identifications obtained for the bacterial isolates, a collective microflora can be constructed for each tissue and for that particular day of ice storage. The microflora of the pink salmon comprises predominantly gram-negative bacteria (Shewan, 1962). The bacterial flora observed on both gills and skin for day 0 of ice storage of fish is a typical representation of the microflora from fresh fish caught from temperate waters. Shewan (1962) reported the presence of *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Vibrio* on the gills of fish. Horsley (1973) found *Moraxella* comprised 32% of the bacterial load on gills in marine caught Atlantic salmon. On day 0, the belly cavity microflora comprised four bacterial species which could be due to the contamination from the intestine and stomach during the gutting process (Shewan, 1962).
As pink salmon storage in ice progressed, there was a change in the microflora (Himelbloom et al., 1994; Himelbloom and Crapo, 1999). The occurrence of *P. immobilis* in the belly cavity can be due to the dislodging on these bacteria from the skin by melting ice (Ryder et al., 1993; Himelbloom et al., 1994) and their introduction in the belly cavity. On day 6 of ice storage, the decrease in *Acinetobacter* (belly cavity) and *P. immobilis* (iced belly) probably indicated the washing effect of the melting ice on the bacterial population. The presence of *P. fluorescens/ putida* at 80% of the total microflora on final day of ice storage in all the tissues reinforces the importance of *Pseudomonas* spp. in the spoilage process (Huss, 1995) of pink salmon stored in ice. Presence of *P. immobilis* and *M. catarrhalis* was observed through out the storage period but its contribution to the spoilage should be investigated.

It can be concluded from the research that the APC increased with duration of ice storage in various tissues of fish. The microflora contributing to the APC of pink salmon was predominantly gram negative flora and *P. fluorescens/putida* were the major species in the APC in the final days of storage.

The Sherlock MIS was useful for rapid identification of seafood isolates from pink salmon. The important step in the extraction of fatty acids is the growth and harvesting of organisms from the late log-phase quadrant. Slow growing bacteria can be streaked on

multiple plates to harvest the required amount of cells. After the results are obtained from Sherlock MIS, it is necessary to make a decision about the identity of the bacteria when more than listing occurs <0.1 SMI unit from the first rank. In such cases, a choice based on the previous knowledge of the sample should be picked. For example, the isolates identified as *P. fluorescens* as the first rank were followed <0.1 SMI by *P. syringae* and *P. chlororaphis*. Careful consideration of the literature and past records have shown that *P. syringae* is a plant pathogen and it would be highly unlikely to be found on the fish caught in the Alaskan waters. Hence *P. syringae* is eliminated and *P. fluorescens* is confirmed as the identity of the previously unknown bacterium. The Sherlock MIS was useful in identifying psychrotrophic seafood bacteria to the species level and can be used as a rapid method for investigating seafood quality.

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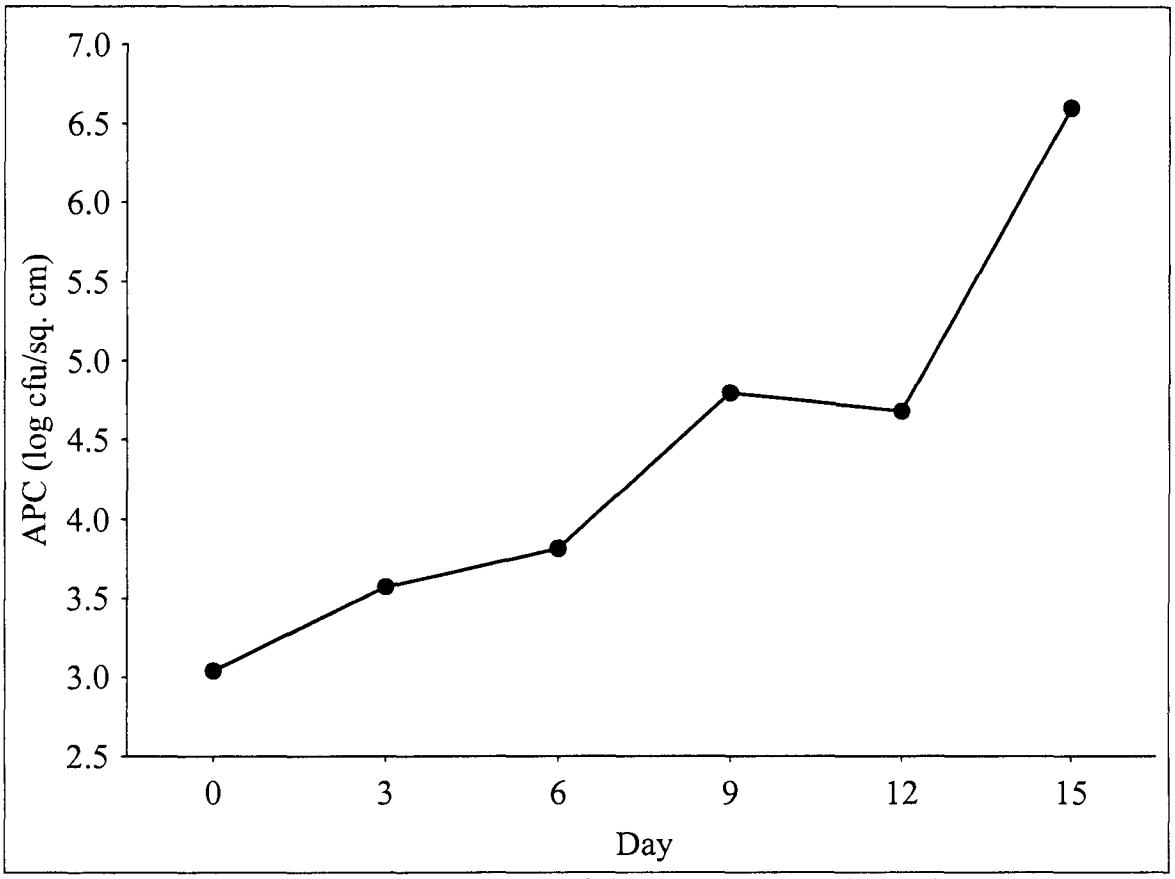


Figure 3.1 Aerobic plate counts (APC; log cfu/sq. cm) of pink salmon skin during ice storage

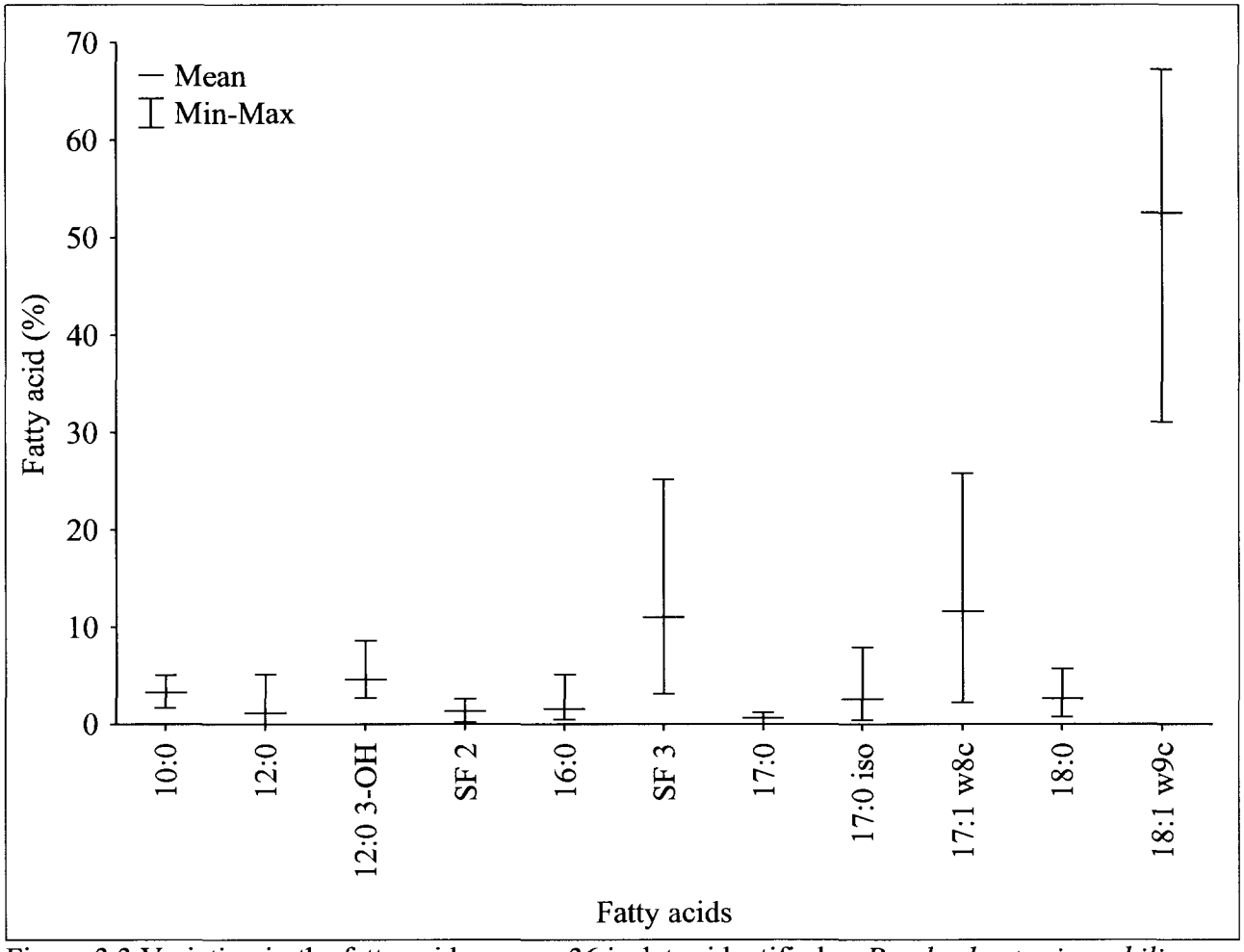


Figure 3.3 Variation in the fatty acids among 36 isolates identified as *Psychrobacter immobilis*
SF 2 (Summed Feature 2) Peak tentatively designated as 12:0 aldehyde or unknown 10.947.
SF 3 (Summed Feature 3) Peak identified as either 16:1 ω6c or 16:1 ω7c

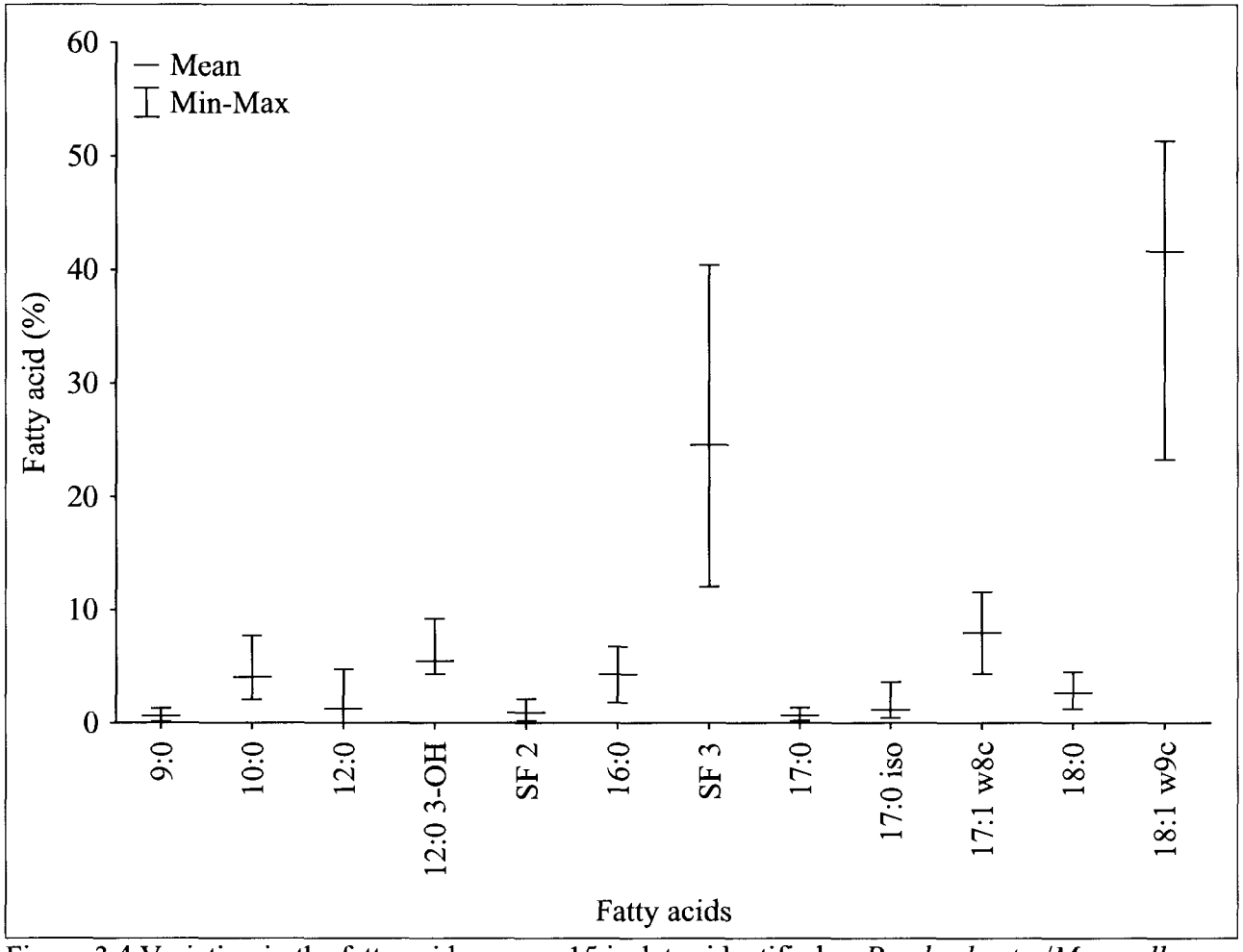


Figure 3.4 Variation in the fatty acids among 15 isolates identified as *Psychrobacter/Moraxella*
*SF 2 and SF 3 refer to Figure 3.3.

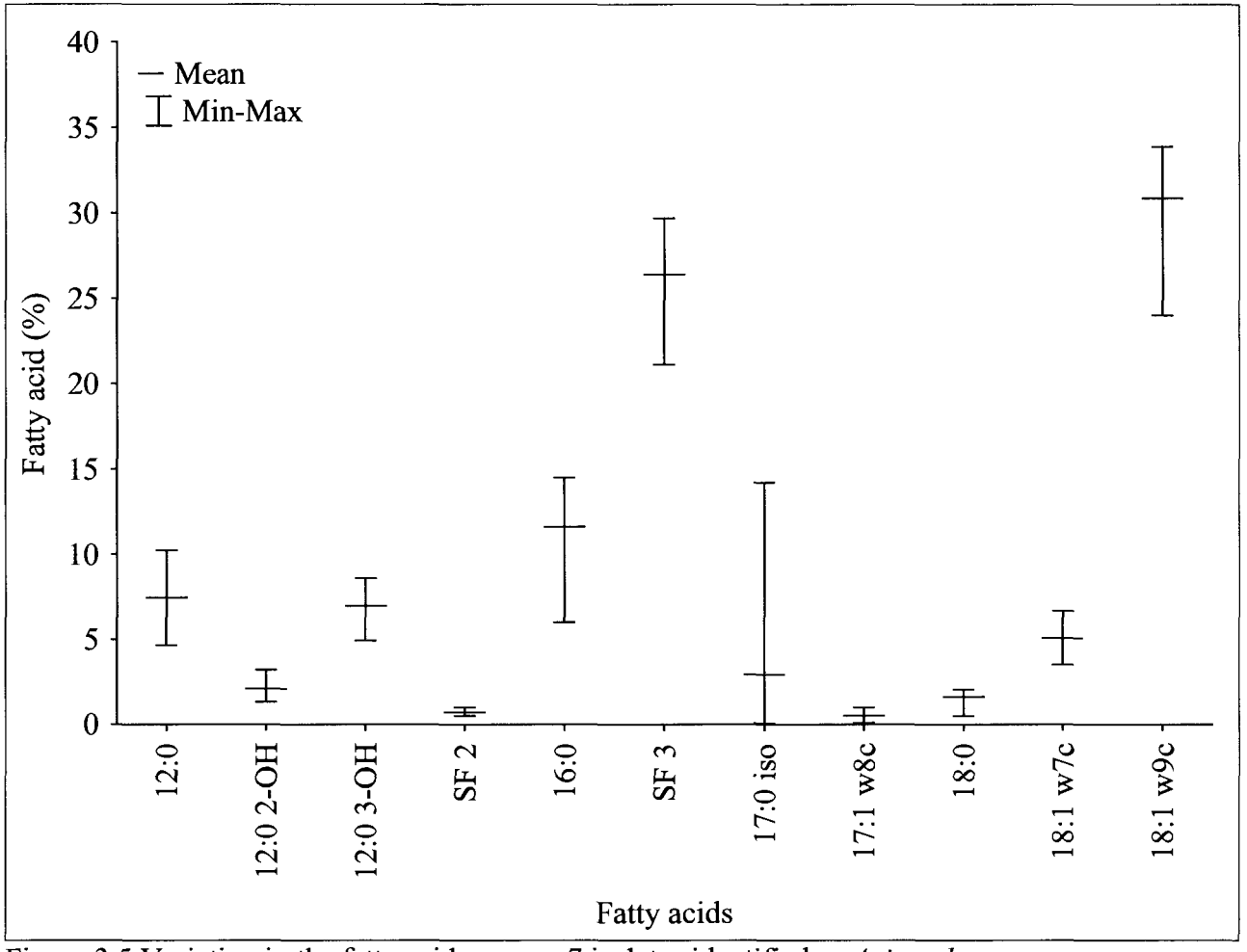


Figure 3.5 Variation in the fatty acids among 7 isolates identified as *Acinetobacter*
*For SF 2 and SF 3 refer to Figure 3.3.

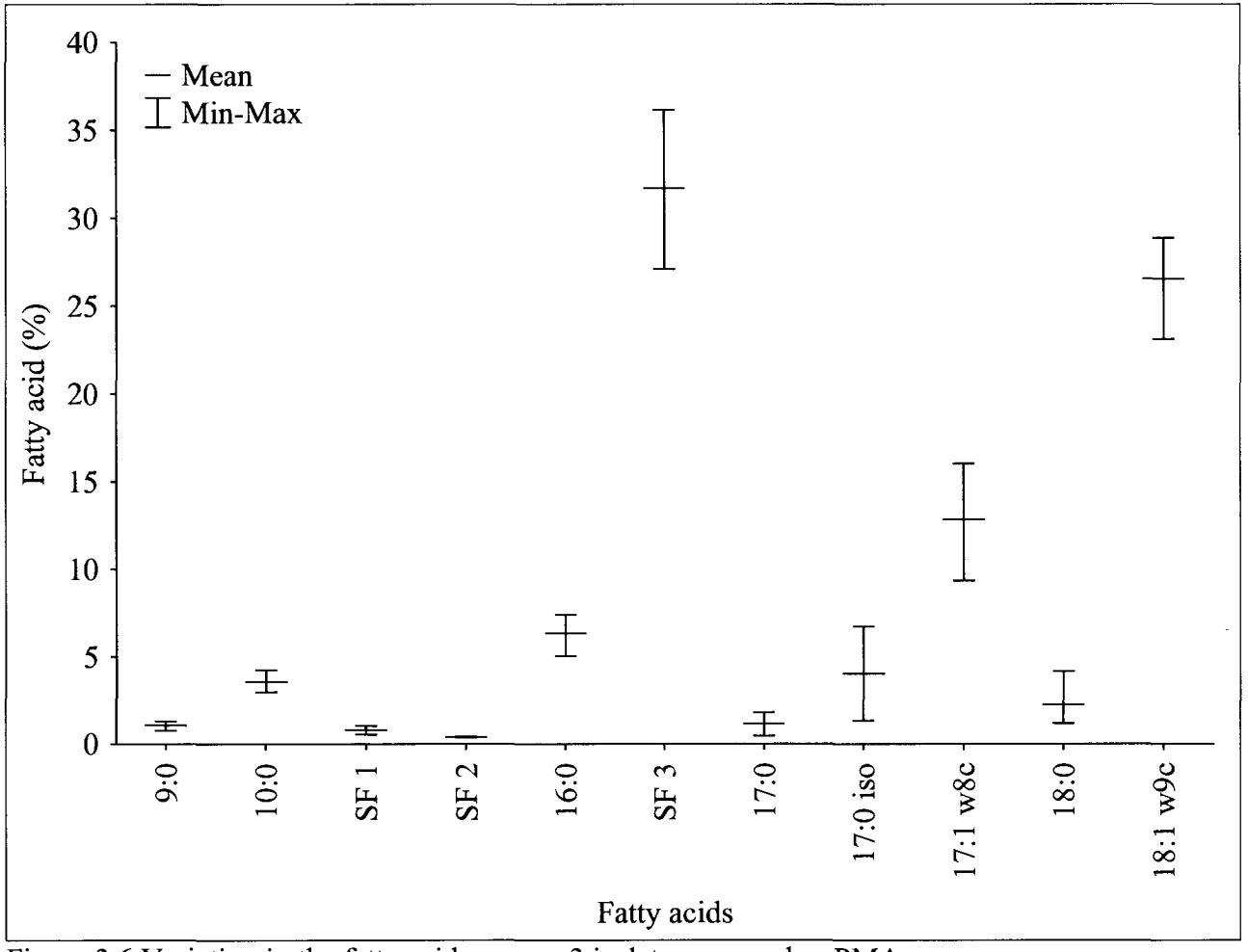


Figure 3.6 Variation in the fatty acids among 3 isolates grouped as PMA
SF 1 (Summed Feature 1) Peak identified as either 15:1 iso OH or 13:0 3-OH
*For SF 2 and SF 3 refer to Figure 3.3.

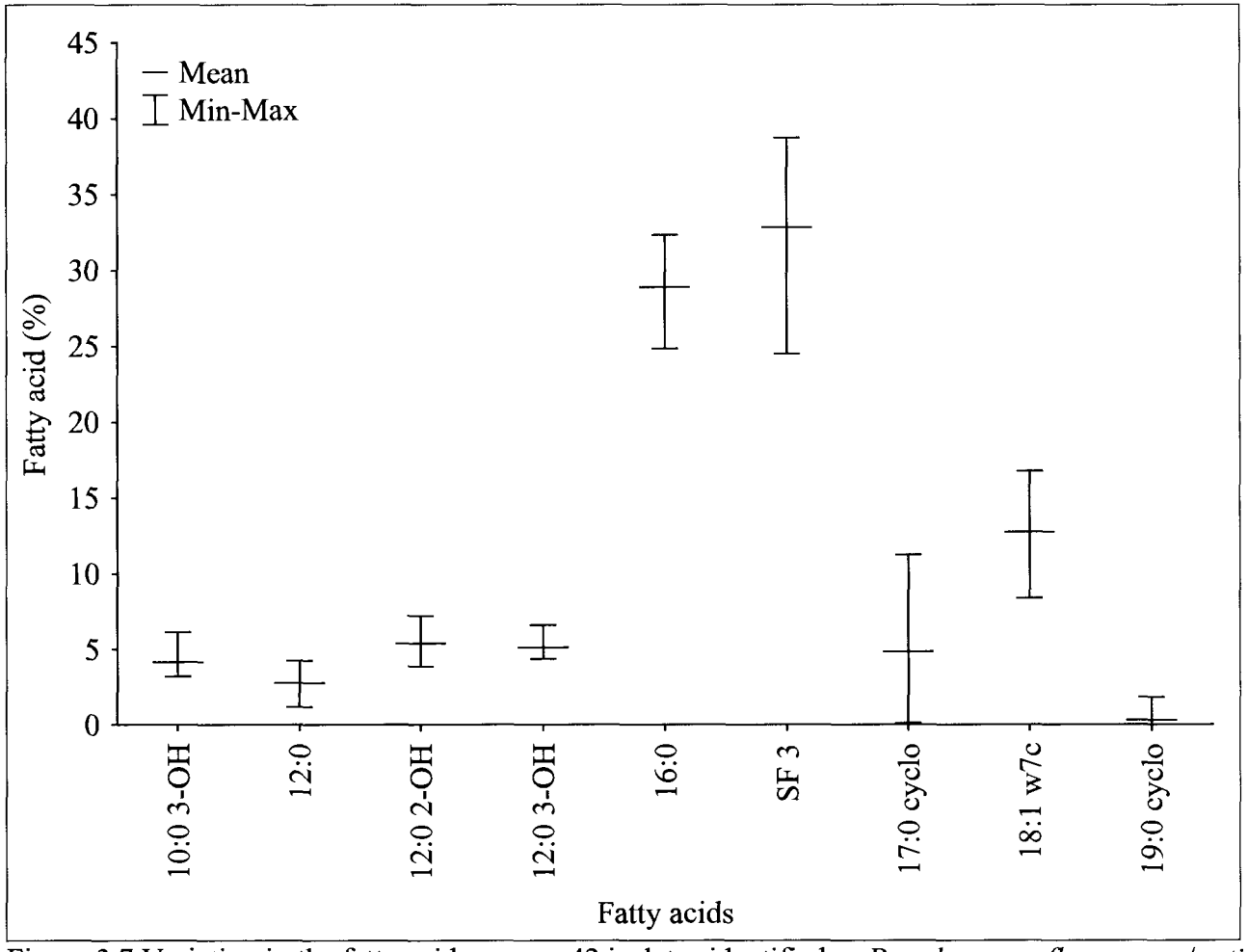


Figure 3.7 Variation in the fatty acids among 42 isolates identified as *Pseudomonas fluorescens/putida* 19:0 cyclopropane ω8c was present in only 60% of isolates identified as *Pseudomonas fluorescens/putida*
*For SF 3 refer to Figure 3.3.

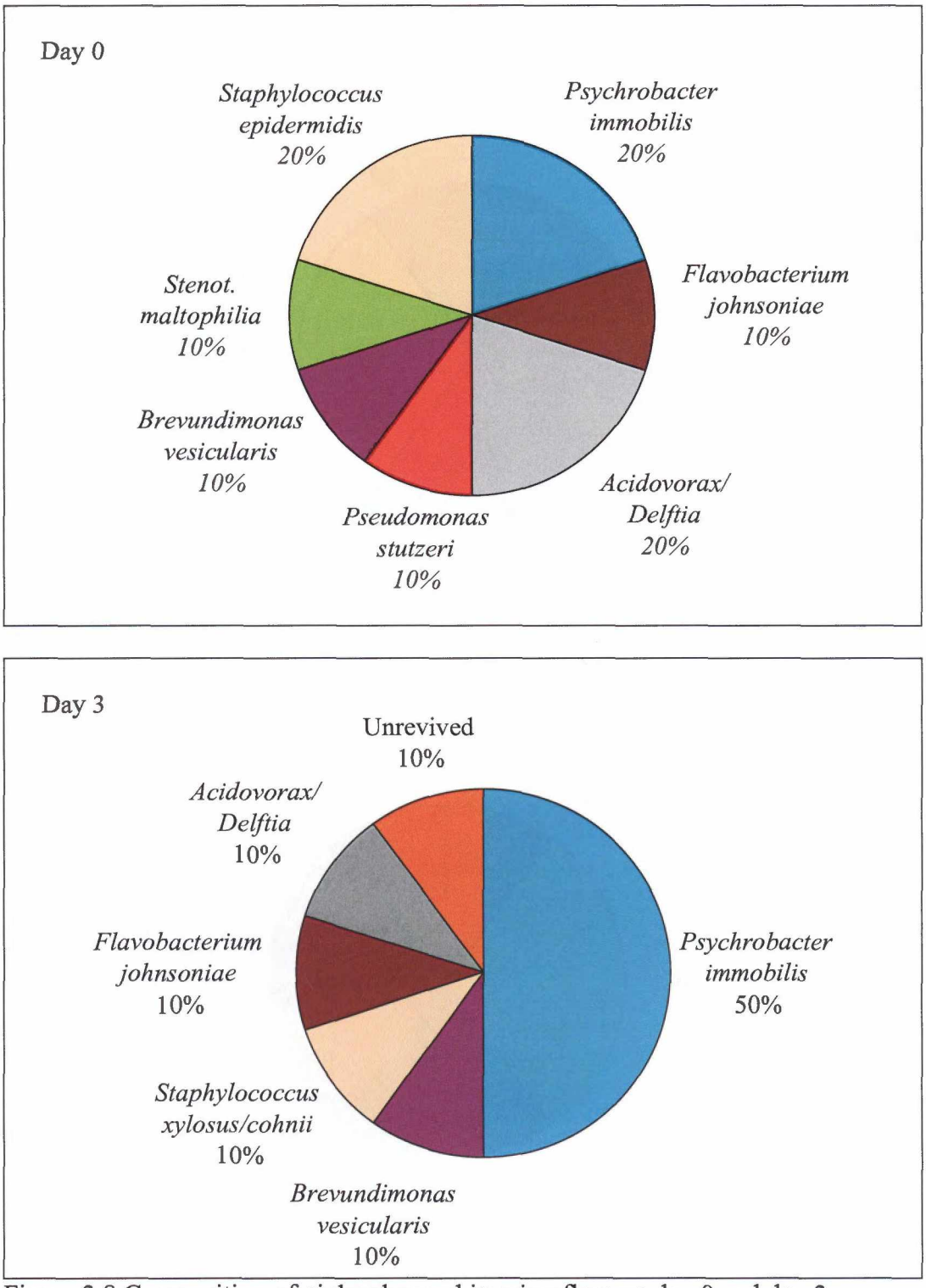


Figure 3.8 Composition of pink salmon skin microflora on day 0 and day 3

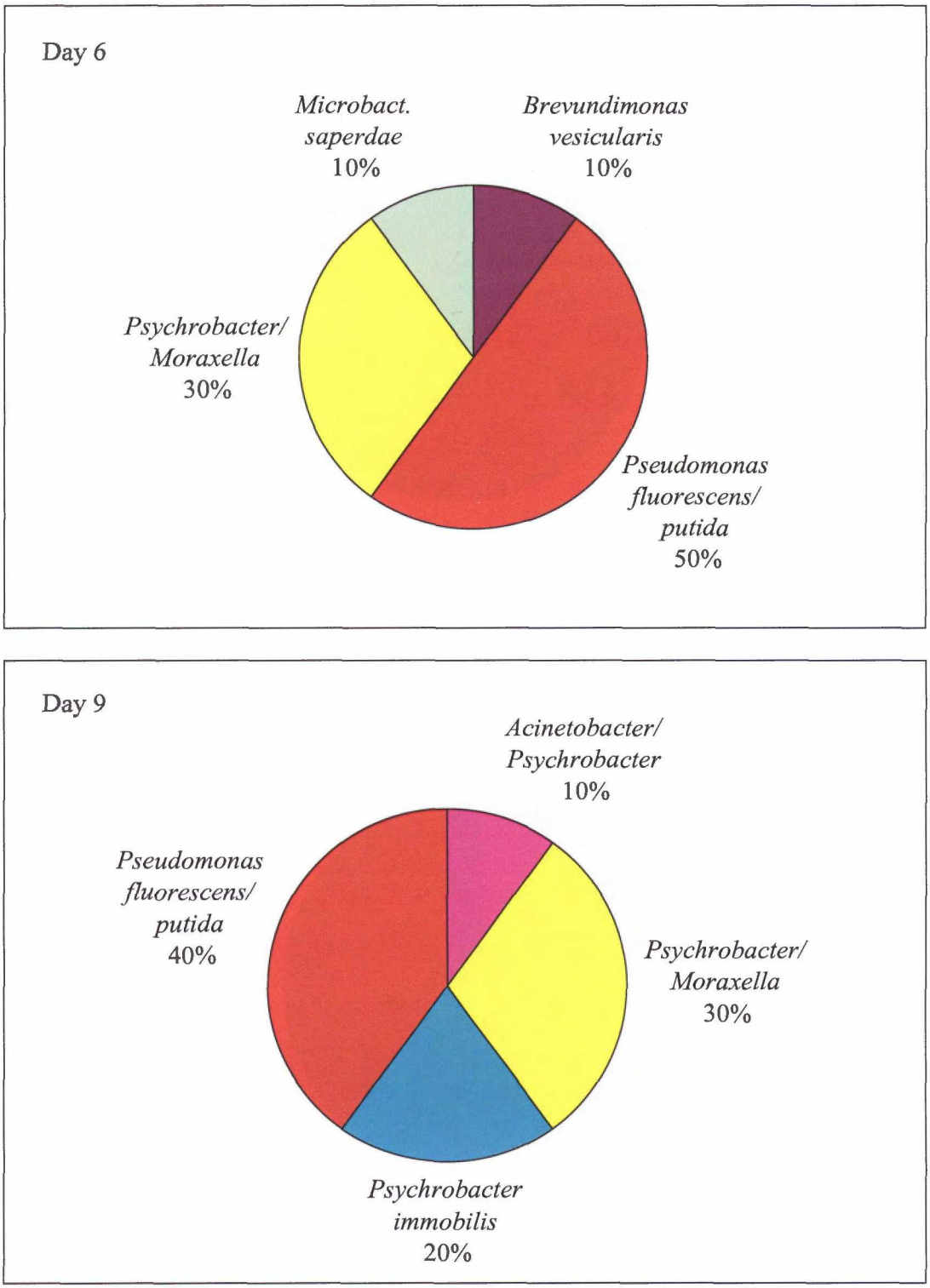


Figure 3.9 Composition of pink salmon skin microflora on day 6 and day 9

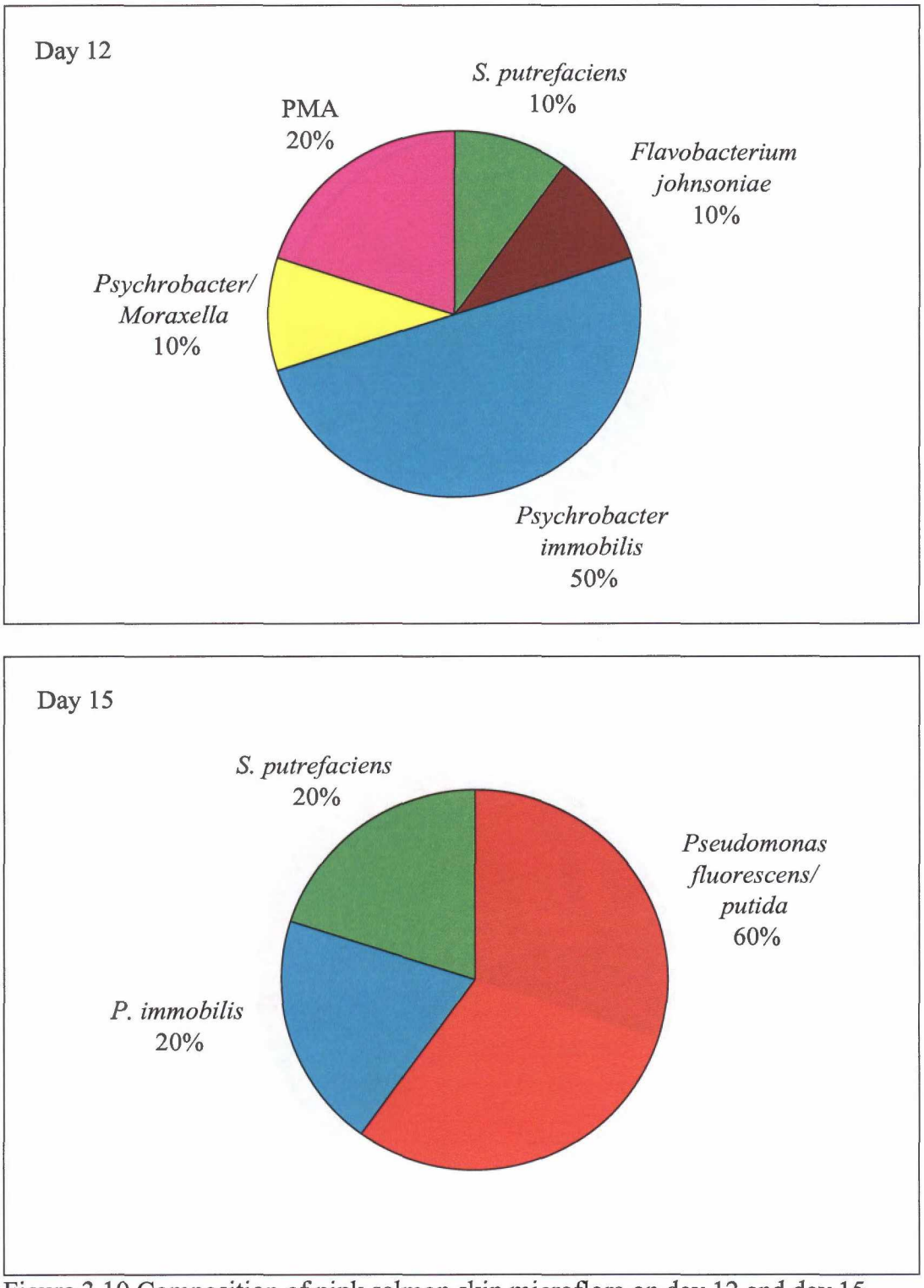


Figure 3.10 Composition of pink salmon skin microflora on day 12 and day 15

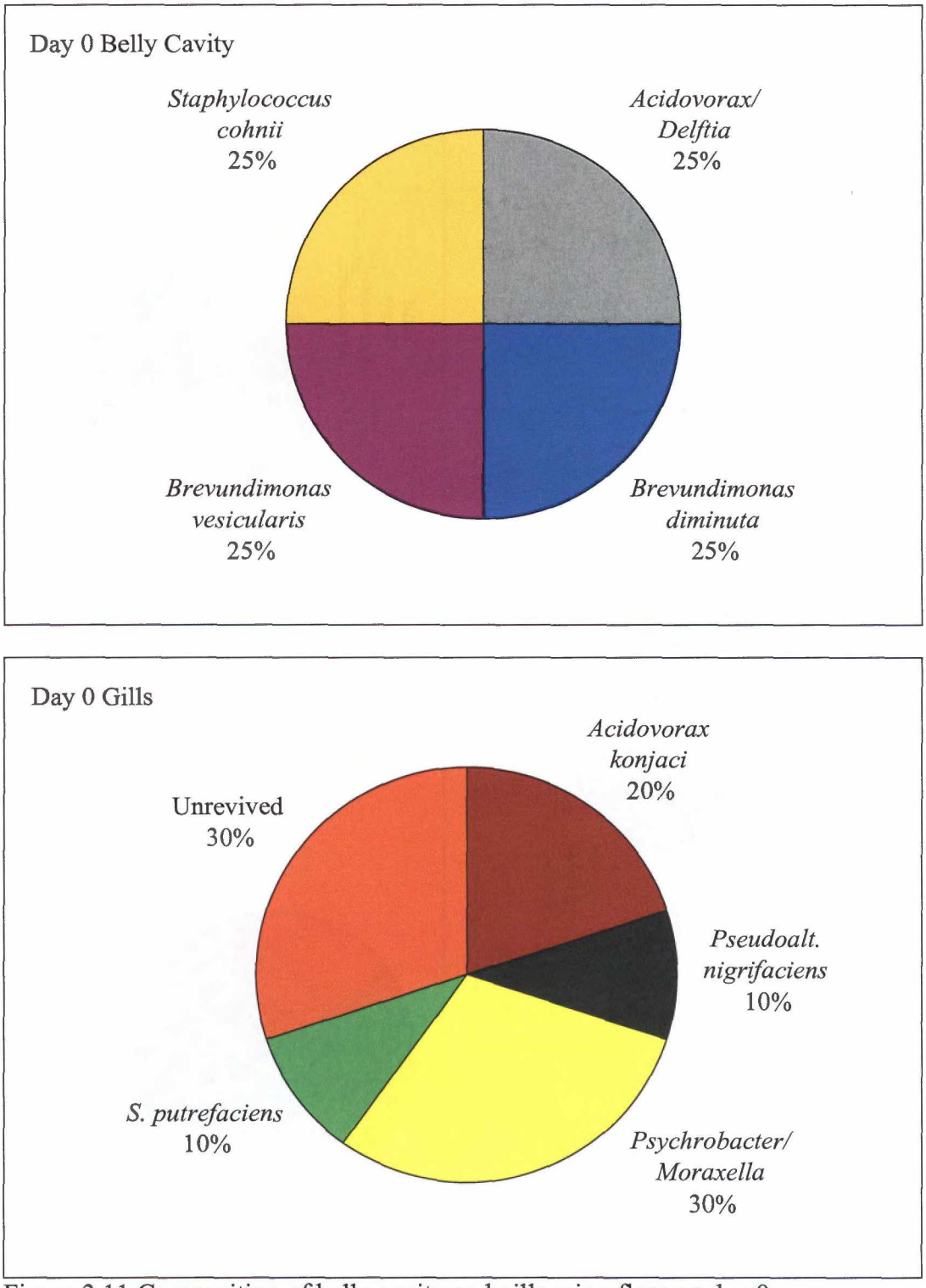
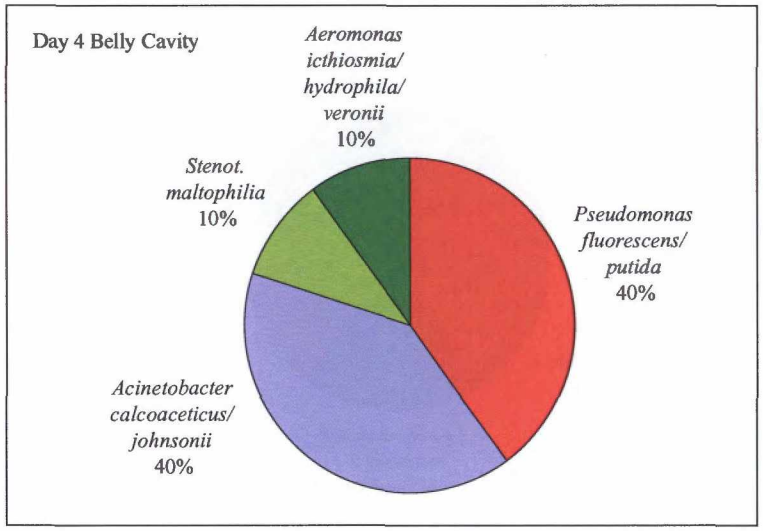
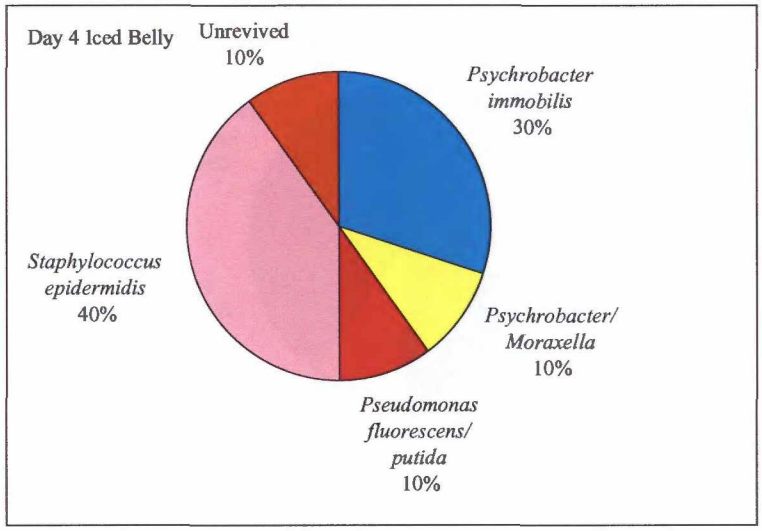


Figure 3.11 Composition of belly cavity and gills microflora on day 0





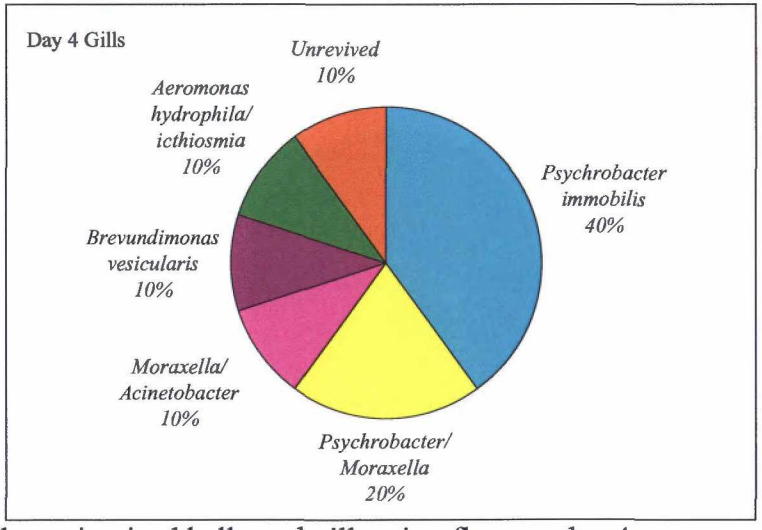
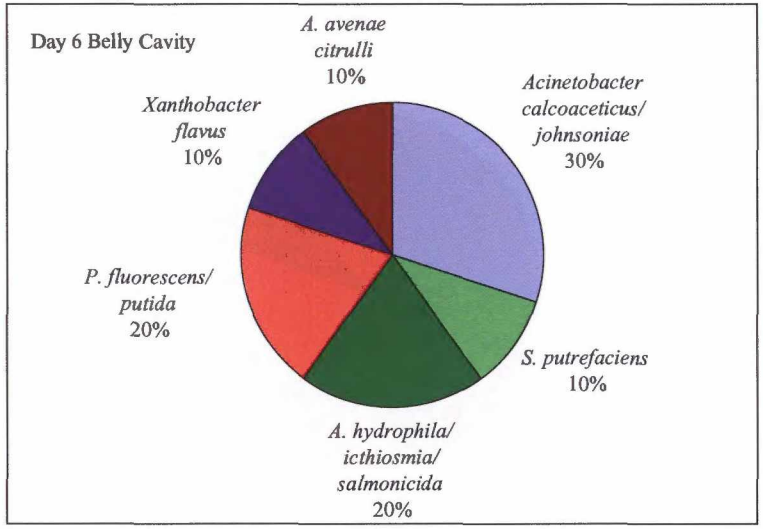
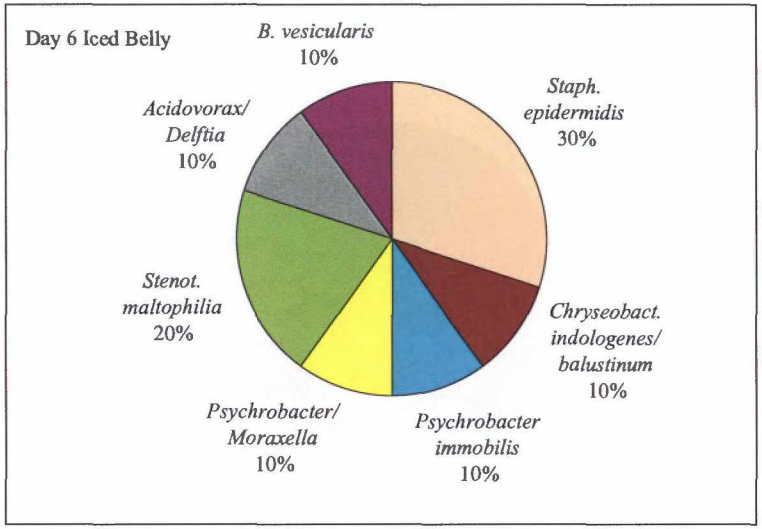


Figure 3.12 Composition of belly cavity, iced belly and gills microflora on day 4





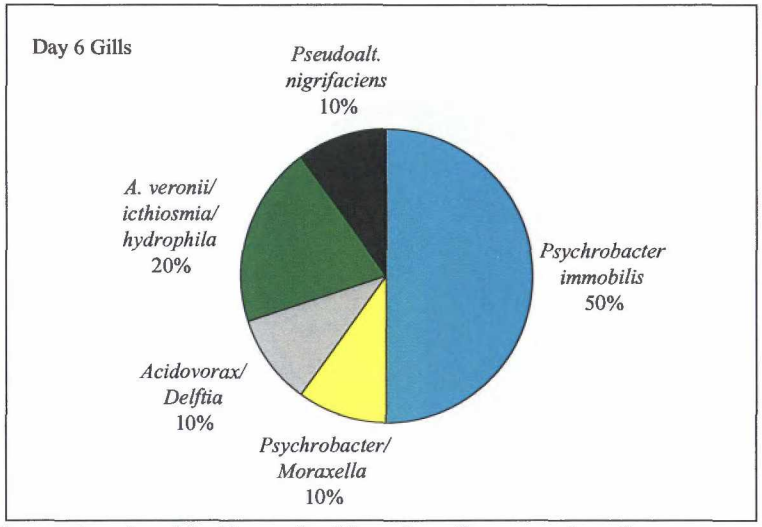
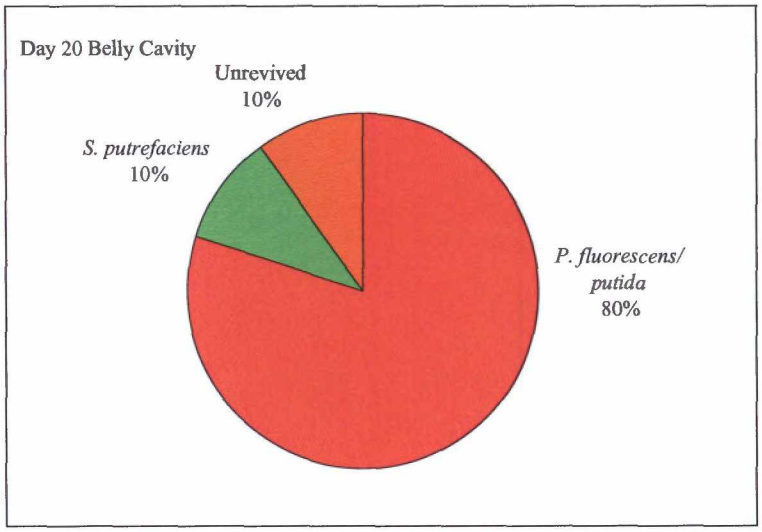
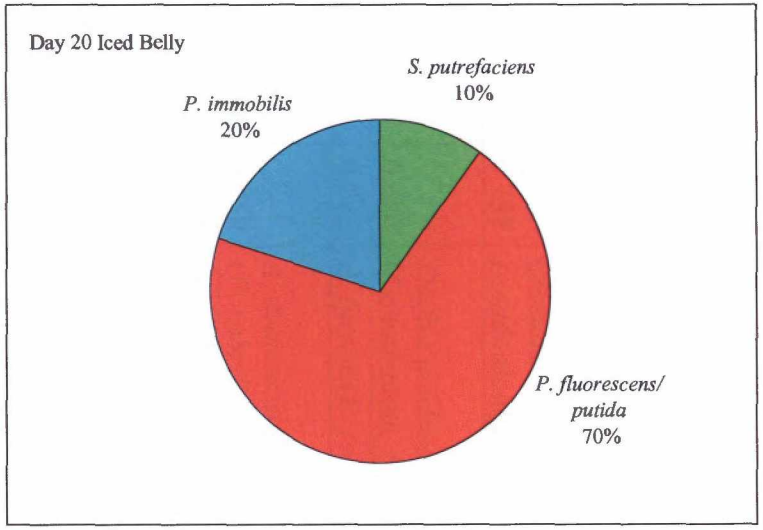


Figure 3.13 Composition of belly cavity, iced belly and gills microflora on day 6





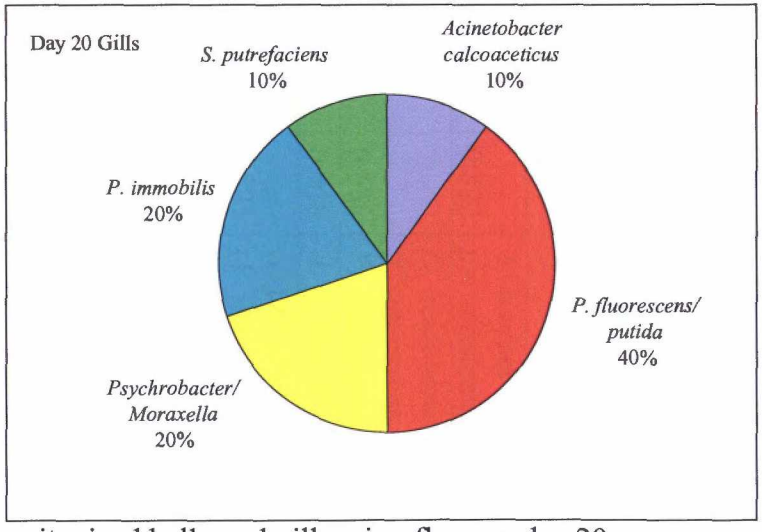


Figure 3.14 Composition of belly cavity, iced belly and gills microflora on day 20

Table 3.1 Similarity index (SMI) and corresponding identification (ID) of two isolates from day 0 salmon skin (SS) and belly (B) as *Acidovorax*

Isolates	Replicate 1		Replicate 2	
	SMI	ID	SMI	ID
4/0/SS2	0.788	*Acidovorax konjaci* (*Pseudomonas pseudoalcaligenes konjaci*)	0.802	*A. konjaci* (*P. pseudoalcaligenes konjaci*)
	0.684	*Delftia acidovorans* (*Comamonas, P. acidovorans*)	0.686	*D. acidovorans* (*Comamonas, P. acidovorans*)
	0.622	*A. facilis* (*P. facilis*)	0.598	*A. facilis* (*P. facilis*)
	0.559	*A. avenae avenae* (*P. rubrilineans*)	0.571	*Hydrogenophaga pseudoflava* (*P. pseudoflava*)
	0.555	*A. avenae avenae* (*P. cattleyae*)		
	0.503	*H. pseudoflava* (*P. pseudoflava*)		
1/0/B2	0.791	*A. konjaci* (*P. pseudoalcaligenes konjaci*)	0.822	*A. konjaci* (*P. pseudoalcaligenes konjaci*)
	0.679	*D. acidovorans* (*Comamonas, P. acidovorans*)	0.658	*D. acidovorans* (*Comamonas, P. acidovorans*)
	0.601	*A. facilis* (*P. facilis*)	0.618	*A. facilis* (*P. facilis*)
	0.57	*H. pseudoflava* (*P. pseudoflava*)	0.583	*H. pseudoflava* (*P. pseudoflava*)
			0.522	*A. avenae avenae* (*P. rubrilineans*)
			0.519	*A. avenae avenae* (*P. rubrilineans*)

Table 3.2 Major fatty acids of the isolates identified under *Acidovorax* and the *Acidovorax-Delftia* group*

Fatty acid	*Acidovorax/ Delftia* (n =4)	*A. avenae/ temperans* (n= 4)	*A. konjaci* (n= 2)
12:0	2.59 ± 0.08	4.13 ± 0.97	3.13
14:0	0.99 ± 0.04	2.83 ± 1.75	1.97
15:0	A	6.36 ± 2.65	T
16:0	32.94 ± 1.60	27.68 ± 1.17	30.52
17:0	A	1.19 ± 0.23	T
17:0 cyclo	3.42 ± 3.23	1.81 ± 0.89	3.28
10:0 3-OH	3.09 ± 0.16	4.57 ± 1.33	2.76
15:1 ω6c	A	1.46 ± 0.17	A
18:1 ω7c	14.74 ± 1.59	7.62 ± 0.97	12.90
SF 3	40.57 ± 3.28	42.38 ± 4.98	43.78

* All values and standard deviations are in percent, T denotes trace amounts (<1%) and A denotes either absent or below detection. cyclo denotes cyclopropane.
Summed Feature (SF) 3 consists of one or both of 16:1 ω6c and 16:1 ω7c, which cannot be identified separately by the Sherlock MIS 4.5.

Table 3.3 Similarity index (SMI) and corresponding identification (ID) of three isolates from salmon skin (SS) and gills (G) under the *Acidovorax/Delftia* group

Isolates		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
6/0/SS2	0.852	*D. acidovorans* (*Comamonas, P. acidovorans*)	0.648	*D. acidovorans* (*Comamonas, P. acidovorans*)
	0.797	*A. konjaci* (*P. pseudoalcaligenes konjaci*)	0.621	*Variovorax paradoxus*-GC subgroup B (*Alcaligenes paradoxus*)
	0.631	*A. avenae avenae* (*P. rubrilineans*)		
	0.576	*A. avenae cattleyae* (*P. cattleyae*)		
	0.556	*A. facilis* (*P. facilis*)		
	0.538	*P. fluorescens* biotype C/ *P. mandelii*		
	0.523	*V. paradoxus*-GC subgroup B (*A. paradoxus*)		
	0.521	*Hydrogenophaga pseudoflava* (*P. pseudoflava*)		
12/3/SS1	0.893	*D. acidovorans* (*Comamonas,P. acidovorans*)	0.885	*D. acidovorans* (*Comamonas,P. acidovorans*)
	0.83	*A. konjaci* (*P. pseudoalcaligenes konjaci*)	0.827	*A. konjaci* (*P. pseudoalcaligenes konjaci*)
	0.653	*A. avenae avenae* (*P. rubrilineans*)	0.658	*A. avenae avenae* (*P. rubrilineans*)
	0.634	*A. facilis* (*P. facilis*)	0.636	*A. facilis* (*P. facilis*)
	0.585	*V. paradoxus*-GC subgroup B (*A. paradoxus*)	0.593	*V. paradoxus*-GC subgroup B (*Alcaligenes paradoxus*)
	0.568	*A. avenae cattleyae* (*P. cattleyae*)	0.566	*A. avenae cattleyae* (*P. cattleyae*)
	0.538	*P. fluorescens* biotype C/ *P. mandelii*	0.546	*P. fluorescens* biotype C/ *P. mandelii*
			0.546	*C. terrigena*
			0.534	*H. pseudoflava* (*P. pseudoflava*)

Table 3.3 contd...

Isolates		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
4/6/G2	0.932	*A. konjaci* (*P. pseudoalcaligenes konjaci*)	Not revived	
	0.897	*D. acidovorans* (*Comamonas, P. acidovorans*)		
	0.883	*A. avenae avenae* (*P. rubrilineans*)		
	0.763	*A. facilis* (*P. facilis*)		
	0.759	*A. avenae cattleyae* (*P. cattleyae*)		
	0.579	*V. paradoxus*-GC subgroup B (*A. paradoxus*)		
20/6/IB1	0.912	*D. acidovorans* (*Comamonas, P. acidovorans*)	0.918	*D. acidovorans* (*Comamonas, P. acidovorans*)
	0.910	*A. avenae avenae* (*P. rubrilineans*)	0.905	*A. konjaci* (*P. pseudoalcaligenes konjaci*)
	0.908	*A. konjaci* (*P. pseudoalcaligenes konjaci*)	0.898	*A. avenae avenae* (*P. rubrilineans*)
	0.799	*A. avenae cattleyae* (*P. cattleyae*)	0.791	*A. avenae cattleyae* (*P. cattleyae*)
	0.677	*A. facilis* (*P. facilis*)	0.664	*A. facilis* (*P. facilis*)

Table 3.4 Similarity index (SMI) and corresponding identification (ID) of four isolates from gills (G) and belly (B) as *Acidovorax avenae citrulli*/*A. temperans*

Isolates		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
53/6/B1	0.609	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)	0.496	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)
5/0/G1	0.6	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)		Not Revived
	0.438	*A. temperans*		
	0.437	*Photobacterium angustum*		
	0.414	*Duganella zoogloeoides*		
	0.403	*A. konjaci* (*P. pseudoalcaligenes konjaci*)		
7/0/G2	0.458	*A. temperans*	0.629	*D. zoogloeoides*
	0.437	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)		
	0.422	*P. angustum*		
	0.409	*D. zoogloeoides*		
	0.276	*A. konjaci* (*P. pseudoalcaligenes konjaci*)		
38/6/G1	0.45	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)	0.473	*A. avenae citrulli* (*P. pseudoalcaligenes citrulli*)

Table 3.5 Similarity index (SMI) and corresponding identification (ID) of four isolates from salmon belly cavity (B) and gills (G) identified as *Aeromonas*

Isolate		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
11/4/B1	0.786	*Aeromonas ichthiosmia/ A. hydrophila*	0.611	*A. salmonicida masoucida*
	0.675	*A. veronii* GC subgroup B (biogroup *sobria*)	0.415	*A. ichthiosmia/ A. hydrophila*
	0.654	*A. hydrophila/A. ichthiosmia/ A. sobria*	0.382	*Vibrio fischeri*
	0.545	*A. caviae*	0.317	*P. angustum*
	0.537	*A.salmonicida masoucida*		
	0.514	*Photobacterium angustum*		
10/6/B2	0.774	*A. ichthiosmia/ A. hydrophila*	0.735	*A. ichthiosmia/ A. hydrophila*
	0.637	*A. hydrophila/A. ichthiosmia/ A. sobria*	0.633	*A. veronii* GC subgroup B (biogroup *sobria*)
	0.632	*Aeromonas veronii* GC subgroup B (biogroup *sobria*)	0.575	*A. hydrophila/A. ichthiosmia/ A. sobria*
	0.552	*P. angustum*	0.561	*A. salmonicida masoucida*
	0.536	*A. caviae*	0.472	*A. caviae*
			0.468	*P. angustum*
47/6/B2	0.825	*A. ichthiosmia/ A. hydrophila*	0.774	*A. icthiosmia/ A. hydrophila*
	0.825	*A. hydrophila/A. ichthiosmia/ A. sobria*	0.73	*A. salmonicida masoucida*
	0.782	*A. caviae*	0.726	*A. hydrophila/A. icthiosmia/ A. sobria*
	0.641	*A. salmonicida masoucida*	0.653	*A. caviae*
	0.569	*P. angustum*	0.612	*A. veronii* GC subgroup B (biogroup *sobria*)
	0.563	*V. fischeri*	0.581	*P. angustum*
	0.544	*A. veronii* GC subgroup B (biogroup *sobria*)	0.578	*V. fischeri*
	0.504	*A. trota/ A. enteropelogenes*	0.465	*V. hollisae*
23/4/G1	0.093	*A. ichthiosmia/ A. hydrophila*	0.080	*A. ichthiosmia/ A. hydrophila*
	0.091	*Pausimonas lemoignei*	0.066	*V. hollisae*

Table 3.6 Fatty acid profiles of isolates identified under genus *Aeromonas**

Fatty acid	*A. hydrophila* (n=4)	*A. veronii* (n=1)
12:0	4.75 ± 0.67	4.35
14:0	1.44 ± 0.97)	2.73
16:0	16.83 ± 5.04	13.48
15:0 iso 3-OH	T	3.70
17:0 iso	1.77 ± 0.64	4.35
18:1 ω7c	14.32 ± 3.93	9.57
SF 2	5.27 ± 3.58	6.20
SF 3	39.97 ± 5.97	44.14
SF 4	T	3.52

* All values and standard deviations are expressed in percent; T denotes trace amounts (<1%).

Summed Features (SF) are assigned to the fatty acid which can not be identified separately by the Sherlock MIS 4.5:

SF 2 consists of one or both of 12:0 aldehyde or unknown 10.947

SF 3 consists of one or both of 16:1 ω6c/ 16:1 ω7c

SF 4 consists of one or both of 10-methyl 16:0 and/or 17:1 iso ω9c.

Table 3.7 Similarity index (SMI) and corresponding identification (ID) of six isolates from salmon skin (SS), belly (B) and gills (G) identified as *Brevundimonas vesicularis/ B. diminuta*

Isolates		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
14/0/SS2	0.793	*Brevundimonas vesicularis* (*P. vesicularis*)	0.794	*B. vesicularis* (*P. vesicularis*)
	0.642	*B. diminuta* (*P. diminuta*)	0.624	*B. diminuta* (*P. diminuta*)
27/3/SS1	0.799	*B. vesicularis* (*P. vesicularis*)	0.801	*B. vesicularis* (*P. vesicularis*)
	0.645	*B. diminuta* (*P. diminuta*)	0.641	*B. diminuta* (*P. diminuta*)
6/6/SS2	0.745	*B. vesicularis* (*P. vesicularis*)		Not revived
2/0/B1	0.912	*B. diminuta* (*P. diminuta*)	0.921	*B. diminuta* (*P. diminuta*)
3/0/B1	0.884	*B. vesicularis* (*P. vesicularis*)	0.87	*B. vesicularis* (*P. vesicularis*)
33/4/G2	0.815	*B. vesicularis* (*P. vesicularis*)	0.73	*B. vesicularis* (*P. vesicularis*)
	0.611	*Halomonas aquamarina* (*Deleya*)	0.671	*B. diminuta* (*P. diminuta*)

Table 3.8 Fatty acid profile of isolates identified as *Brevundimonas vesicularis* and *B. diminuta**

Fatty acid	*B. vesicularis* (n=5)	*B. diminuta* (n=1)
14:0	2.14 ± 1.69	1.38
15:0	1.48 ± 1.12	1.07
16:0	20.18 ± 2.00	26.79
18:0	2.16 ± 0.39	1.91
19:0 cyclo ω8c	0.40 ± 0.38	5.93
12:0 3-OH	2.77 ± 0.60	2.29
17:1 ω8c	1.37 ± 1.41	0.73
18:1 ω7c	58.51 ± 1.63	52.41
11-methyl 18:1 ω7c	1.16 ± 0.69	1.92
SF 3	6.70 ± 1.86	1.18

* All values and standard deviations are expressed in percent. Cyclo denotes cyclopropane. Summed Feature (SF) 3 consists of one or both of 16:1 ω6c and 16:1 ω7c which cannot be identified separately by the Sherlock MIS 4.5.

Table 3.9 Similarity index (SMI) and corresponding identification (ID) of five isolates from salmon skin (SS), gills (G) and iced belly (IB) identified as *Flavobacterium* and *Chryseobacterium*

Isolates		Replicate 1		Replicate 2
	SMI	ID	SMI	ID
7/0/SS2	0.613	*F. johnsoniae* (*Cytophaga johnsonae*)	0.589	*F. johnsoniae* (*C. johnsonae*)
7/3/SS2	0.613	*F. johnsoniae* (*C. johnsonae*)	0.612	*F. johnsoniae* (*C. johnsonae*)
6/12/SS2	0.162	*F. johnsoniae* (*C. johnsonae*)	0.158	*F. johnsoniae* (*C. johnsonae*)
	0.113	*Zobellia uliginosa* (*.Cytophaga*)	0.139	*Z. uliginosa* (*Cytophaga*)
38/6/G2	0.531	*Chryseobacterium meningosepticum* (*Flavobacterium*)	0.401	*C. meningosepticum* (*Flavobacterium*)
			0.332	*Pedobacter hiparinus* (*Cytophaga heparina*, *Sphingobacterium*)
1/6/IB2	0.941	*Chryseobacterium indologenes* (*Flavobacterium*)	0.905	*C. indologenes* (*Flavobacterium*)
	0.817	*Chryseobacterium balustinum* (*Flavobacterium*)	0.722	*C. balustinum* (*Flavobacterium*)

Table 3.10 Fatty acid profile of isolates identified as *Flavobacteirum* and *Chryseobacterium**

Fatty acid	*F. johnsoniae* (n=3)	*C. indologenes* (n=1)	*C. meningosepticum* (n=1)
14:0	1.10 ± 0.71	T	1.51
15:0	2.75 ± 1.62	T	1.27
16:0	3.27 ± 2.12	1.16	1.07
14:0 iso	T	T	1.36
15:0 iso	24.28 ± 1.03	33.56	39.40
17:0 iso	T	1.07	A
15:0 anteiso	3.87 ± 1.22	T	T
15:0 iso 3-OH	9.83 ± 3.76	3.07	3.40
16:0 iso 3-OH	1.90 ± 0.18	T	T
17:0 iso 3-OH	9.83 ± 1.75	21.50	16.29
15:1 iso G	2.99 ± 2.38	T	A
16:0 3-OH	5.53 ± 3.44	1.28	2.40
15:1 ω6c	2.98 ± 1.80	T	1.24
16:1 ω5c	1.17 ± 0.41	T	2.07
17:1 ω6c	1.81 ± 1.08	A	A
SF 3	18.95 ± 6.20	12.20	24.31
SF 4	3.77 ± 0.98	23.77	1.85

* All values and standard deviations expressed in percent, T denotes trace amounts (<1%) and A denotes either absent or below detection. G indicates that the position of the double bond is unknown. For SF 3 and 4 refer to Table 3.6 footnote.

Table 3.11 Similarity index (SMI) of the 36 isolates from salmon skins (SS), gills (G) and iced bellies (IB) identified as *Psychrobacter immobilis*

Isolates	Replicate 1	Replicate 2
15/0/SS2	0.671	0.680
13/0/SS2	0.623	NR
10/3/SS1	0.574	0.572
26/3/SS1	0.334	0.325
5/3/SS2	0.529	0.519
26/3/SS2	0.778	0.772
27/3/SS2	0.565	0.560
37/6/SS1	0.877	0.905
4/9/SS1	0.745	0.372
21/9/SS2	0.53	NR
17/12/SS1	0.061	NR
1/12/SS1	0.509	0.303
17/12/SS2	0.266	0.086
22/12/SS2	0.82	0.862
26/12/SS2	0.596	0.607
25/15/SS1	0.747	0.765
15/15/SS2	0.918	0.907
4/0/G1	0.468	0.497
2/0/G1	0.321	0.756
26/4/G1	0.486	0.663
82/4/G1	0.708	0.687
31/4/G2	0.549	0.613
45/4/G2	0.707	0.716
2/6/G1	0.540	0.790
33/6/G1	0.823	0.864
8/6/G2	0.739	0.199
18/6/G2	0.511	0.577
34/6/G2	0.911	0.913
5/20/G2	0.716	0.686
10/20/G2	0.873	0.853
6/4/IB1	0.807	0.802
1/4/IB1	0.206	0.622
3/4/IB2	0.831	0.440
11/6/IB2	0.642	0.638
29/20/IB1	0.425	NR
31/20/IB2	0.922	NR

NR denote not revived from the frozen stock

Table 3.12 Similarity index (SMI) and corresponding identification (ID) of seven isolates from salmon skin (SS), gills (G) and iced belly (IB) identified as *Acinetobacter*

Isolates	SMI	Replicate 1	SMI	Replicate 2
3/4/B2	0.589	*Acinetobacter calcoaceticus*	0.696	*A. calcoaceticus*
	0.358	*A. johnsonii*	0.543	*A. johnsonii*
5/4/B2	0.274	*A. calcoaceticus*	0.635	*A. calcoaceticus*
23/4/B2	0.176	*A. calcoaceticus*	0.693	*A. johnsonii*
			0.632	*A. calcoaceticus*
33/4/B1	0.764	*A. calcoaceticus*	0.774	*A. calcoaceticus*
15/6/B1	0.633	*A. calcoaceticus*	0.719	*A. johnsonii*
	0.523	*A. johnsonii*		
20/6/B1	0.048	*A. calcoaceticus*	0.034	*A. calcoaceticus*
53/6/B2	0.767	*A. calcoaceticus*	0.654	*A. calcoaceticus*
			0.438	*Moraxella catarrhalis* (*Branhamella catarrhalis*)

Table 3.13 Identification of four isolates as *Stenotrophomonas maltophilia*

Isolates	SMI	Replicate 1	SMI	Replicate 2
9/0/SS2	0.350	*Stenotrophomonas maltophilia* (*Xanthomonas*, *Pseudomonas*)	0.351	*S. maltophilia* (*Xanthomonas*, *Pseudomonas*)
	0.273	*Xanthomonas campestris campestris*	0.277	*X. campestris campestris*
12/4/B1	0.794	*S. maltophilia* (*Xanthomonas*, *Pseudomonas*)	Not revived	
28/6/IB1	0.584	*S. maltophilia* (*Xanthomonas*, *Pseudomonas*)	0.576	*S. maltophilia* (*Xanthomonas*, *Pseudomonas*)
26/6/IB1	0.626	*S. maltophilia* (*Xanthomonas*, *Pseudomonas*)	Not revived	

Table 3.14 Identification of nine isolates as *Staphylococcus epidermidis*

Isolate	SMI	Replicate 1	SMI	Replicate 2
16/0/SS2	0.792	*S. epidermidis* (GC subgroup C)	0.383	*S. aureus* (GC subgroup G)
	0.500	*S. cohnii cohnii* (GC subgroup A)	0.323	*S. simulans* (GC subgroup B)
			0.282	*S. simulans* (GC subgroup A)
11/0/SS2	0.822	*S. epidermidis* (GC subgroup C)	0.812	*S. epidermidis* (GC subgroup C)
29/3/SS1	0.597	*S. xylosus*	Not Revived	
	0.546	*S. cohnii cohnii* (GC subgroup A)		
	0.398	*S. cohnii urealyticus*		
	0.387	*S. simulans* (GC subgroup A)		
2/0/B2	0.902	*S. cohnii cohnii* (GC subgroup A)	0.751	*S. cohnii cohnii* (GC subgroup A)
	0.557	*S. simulans* (GC subgroup A)	0.574	*S. simulans* (GC subgroup A)
	0.378	*S. saprophyticus*	0.566	*S. saprophyticus*
			0.514	*S. xylosus*
2/4/IB2	0.733	*Staphylococcus epidermidis* (GC subgroup C)	0.689	*S. warneri*
	0.591	*S. epidermidis* (GC subgroup B)	0.672	*S. epidermidis* (GC subgroup B)
			0.623	*S. epidermidis* (GC subgroup A)
3/4/IB1	0.599	*S. epidermidis* (GC subgroup B)	0.447	*S. epidermidis* (GC subgroup C)
	0.457	*S. epidermidis* (GC subgroup C)	0.282	*S. cohnii cohnii* (GC subgroup A)
2/4/IB1	0.805	*S. epidermidis* (GC subgroup A)	0.576	*S. epidermidis* (GC subgroup B)
	0.749	*S. epidermidis* (GC subgroup B)	0.460	*S. aureus*
	0.624	*S. warneri*		

Table 3.14 contd...

Isolate	SMI	Replicate 1	SMI	Replicate 2
11/4/IB1	0.699	*S. epidermidis* (GC subgroup C)	0.670	*S. epidermidis* (GC subgroup C)
	0.628	*S. epidermidis* (GC subgroup B)	0.662	*S. epidermidis* (GC subgroup B)
	0.437	*S. epidermidis* (GC subgroup A)	0.446	*S. epidermidis* (GC subgroup A)
			0.433	*S. warneri*
16/6/IB1	0.736	*S. epidermidis* (GC subgroup B)	0.51	*S. epidermidis*
	0.706	*S. warneri*	0.448	*S. cohnii cohnii* (GC subgroup A)
	0.685	*S. aureus* (GC subgroup C)	0.415	*S. xylosus*
	0.633	*S. epidermidis* (GC subgroup A)	0.396	*S. aureus* (GC subgroup G)
	0.478	*S. aureus* (GC subgroup E)		
	0.462	*S. aureus* (GC subgroup D)		
7/6/IB1	0.759	*S. epidermidis* (GC subgroup C)	0.676	*S. epidermidis* (GC subgroup C)
	0.605	*S. epidermidis* (GC subgroup B)	0.546	*S. aureus* (GC subgroup C)
	0.578	*S. epidermidis* (GC subgroup A)	0.539	*S. epidermidis* (GC subgroup A)
	0.532	*S. warneri*	0.522	*S. epidermidis* (GC subgroup B)
	0.532	*S. aureus* (GC subgroup C)	0.508	*S. warneri*
19/6/IB1	0.748	*S. epidermidis* (GC subgroup B)	0.712	*S. epidermidis* (GC subgroup C)
	0.745	*S. epidermidis* (GC subgroup A)	0.492	*S. aureus* (GC subgroup G)
	0.694	*S. warneri*		
	0.657	*S. aureus* (GC subgroup C)		
	0.640	*S. epidermidis* (GC subgroup C)		

Table 3.15 Identification of four isolates as *Microbacterium*, *Xanthobacter* and *Pseudoalteromonas*

Isolates	SMI	Replicate 1	SMI	Replicate 2
36/6/SS2	0.549	*Microbacterium saperdae* (*Aureobacterium*, *Curtobacterium*)	0.647	*M. saperdae* (*Aureobacterium*, *Curtobacterium*)
	0.334	*Kocuria kristinae*	0.596	*K. kristinae*
			0.535	*M. esteraromaticum*
			0.466	*M. barkeri* (*Aureobacterium*, *Corynebacterium*)
58/6/B1	0.762	*Xanthobacter flavus*	0.898	*Xanthobacter flavus*
	0.618	*Ochrobactrum anthropi* (*Achromobacter* Vd, CDC group Vd)	0.889	*M.mesophilicum/ radiotolerans* (48h, *Pseudomonas*)
			0.837	*M. zatmanii* (48h)
			0.793	*M. rhodesianum* (48h)
			0.697	*R. sphaeroides* (*Rhodopseudomonas sphaeroides*)
			0.591	*O. anthropi* (*Achromobacter* Vd, CDC group Vd)
			0.548	*M. organophilum/ fujisawaense* (48h)
10/0/G2	0.807	*Pseudoalteromonas nigrifaciens*	Not Revived	
	0.641	*P. tetraodonis*		
10/6/G1	0.725	*P. nigrifaciens*		
	0.629	*P. tetraodonis*		

Table 3.16 Fatty acid profile of isolates identified as *Stenotrophomonas maltophilia* (n=4), *Xanthobacter flavus* (n=1), *Pseudoalteromonas nigrifaciens* (n=2), *Shewanella putrefaciens* (n=7), *Staphylococcus epidermidis* (n=9), *Microbacterium/Kocuria* (n=1)*

	S. maltophilia	*X. flavus*	*P. nigri-faciens*	*S. putre-faciens*	*S. epidermidis*	*M. saperdae*
12:0		A	3.64	2.48 ± 0.81	A	A
14:0	3.55 ± 0.56	A	2.64	1.48 ± 0.23	2.65 ± 1.93	T
15:0	T	A	4.44	7.05 ± 2.28	A	T
16:0	7.33 ± 1.98	1.24	22.10	7.10 ± 1.42	3.27 ± 1.45	4.17
17:0	T	T	1.59	2.22 ± 0.59	T	T
18:0	T	4.14	T	A	10.19 ± 2.15	T
20:0	A	A	A	A	19.11 ± 2.46	A
11:0 iso	4.38 ± 0.30	A	A	A	A	A
13:0 iso	A	A	A	8.00 ± 1.92	A	A
14:0 iso	T	A	A	2.52 ± 0.81	4.11 ± 1.88	1.32
15:0 iso	26.19 ± 5.12	A	T	11.87 ± 1.92	11.98 ± 1.77	2.98
11:0 3-OH	T	A	1.00		A	A
12:0 3-OH	3.07 ± 1.73	T	9.24	1.72 ± 0.24	A	A
12:1 3-OH	T	A	T		A	A
16:0 iso	1.40 ± 0.47	A	T	A	1.25 ± 0.54	21.38
17:0 iso	2.43 ± 0.39	A	1.20	A	4.61 ± 1.65	T
19:0 iso	T	A	A	A	3.61 ± 2.55	A
11:0 iso 3-OH	2.39 ± 0.57	A	T		A	A
13:0 iso 3-OH	3.47 ± 0.85	A	T	2.49 ± 1.39	A	2.72

Table 3.16 contd...

	S. maltophilia	*X. flavus*	*P. nigri-faciens*	*S. putre-faciens*	*S. epidermidis*	*M. saperdae*
15:0 anteiso	11.14 ± 3.76	A	T		30.84 ± 1.68	54.41
17:0 anteiso	T	A	T	A	3.87 ± 1.08	11.03
19:0 anteiso	A	A	A	A	1.86 ± 1.12	A
15:1 iso F	1.82 ± 0.22	A	T	A	A	A
15:1 ω6c	A	A	A	1.19 ± 0.50	A	A
15:1 ω8c	T	A	3.40	1.43 ± 0.67	A	A
16:1 ω9c	3.74 ± 1.02	A	A	A	A	A
17:1 ω8c	T	A	3.87	13.38 ± 4.01	A	A
18:1 ω7c	1.01 ± 0.12	87.67	2.14	2.71 ± 1.19	T	A
18:1 ω9c	1.42 ± 0.85	A	A	1.43 ± 0.39	A	A
19:0 cyclo ω8c	A	4.66	A	A	A	A
SF 1	A	A	A	1.88 ± 0.68	A	A
SF 2	A	A	A	1.87 ± 0.50	A	A
SF 3	11.51 ± 2.18	T	37.00	19.83 ± 4.37	A	A
SF 4	6.10 ± 2.10	A	A	A	A	A
unknown 11.825	1.30 ± 0.31	A	T	A	A	A

*All values and standard deviations expressed in percent, T denotes fatty acids < 1.0% and A denotes fatty acid was either absent or below detection. Cyclo denotes cyclopropane fatty acid. For *S. epidermidis*, the standard deviation for 19:0 iso was approaching the value because the minimum value was 1.44% and maximum value was 8.43%. SF 1 is Summed Feature 1 consist of one or both of 15:1 iso OH and 13:0 3-OH which could not be distinguished by Sherlock MIS. For SF 2- 4 refer to Table 3.6. Unknown 11.825, fatty acid could not be identified by the Sherlock MIS.

Summary

Time-consuming and laborious classical methods of bacterial identification are being replaced by rapid methods in microbiology. Sherlock MIS is one such system designed to rapidly identify bacteria based on their cellular fatty acid profile. This system has been used in clinical as well as environmental microbiology but its reported use in food microbiology is limited and almost lacking in seafood microbiology. Hence this thesis project was undertaken to understand the ability of Sherlock MIS to identify the psychrotrophic bacteria associated with seafood. In addition, the cellular fatty acid data obtained was analyzed. After the confirming the identification ability of the system, a spoilage study was conducted to track the microbial progression in various tissues of the pink salmon stored in ice.

Regarding the identification ability of Sherlock MIS this study showed:

- Sherlock MIS had a greater ability to identify standard ATCC strains as compared to the FITCCC strains isolated from seafoods.

- Identification ability of Sherlock MIS for the isolates belonging to Enterobacteriaceae was found to be not reproducible among replicates for the same isolate.

- Closely related species like *Pseudomonas fluorescens* and *P. putida* could not be easily distinguished from each other and hence were grouped together as *P. fluorescens/putida*.

- In cases where there was more than one identification within the 0.1 SMI range of the first rank, a general perception of the sample could help eliminate the identifications which might not be related to the sample.

- Since the FITCCC strains, *Alteromonas* spp., *Moraxella* sp. and *Flavobacterium* sp. were identified as *Shewanella putrefaciens*, *Psychrobacter immobilis* and *Myroides odoratus* respectively by Sherlock MIS, it can be concluded that the database of the collection needs to be updated to accommodate latest taxonomic changes.

- Bacterial taxonomy has been seen to be rapidly changing. New species are continuously identified, various species are moved to a different genera or novel genera, and genus is emended or upgraded to family level. Hence in addition to the dependence on commercial identification systems, the user must be aware of the current taxonomic knowledge of the bacteria under study.

Tracking the microbial progression in ice stored pink salmon in this study showed:

- Fresh pink salmon caught in cold temperate waters have low aerobic plate count (APC).

- APC increase to about 7 log cfu/sq. cm in 15 days on salmon skin while it reaches 8 log cfu/sq. cm or /g in 20 days in iced or uniced belly and gills.

- This study reinforces the scientific literature that gram-negative bacteria are isolated from temperate water fish during ice storage and confirms that the SSO for pink salmon spoilage is *Pseudomonas* species.

- Among various bacterial species identified, gram-negative coccobacilli *P. immobilis*, *Acinetobacter calcoaceticus/johnsonii*, *Psychrobacter/Moraxella* and *Psychrobacter/Moraxella/Acinetobacter* group together formed an interesting set of bacteria that needs further research about their role(s) in seafood quality.

- Higher numbers of *Pseudomonas fluorescens/putida* and few *Shewanella putrefaciens* in the microflora at the end of storage study indicate that the former is the major contributor to pink salmon spoilage under ice storage.

- In addition to these trends, some isolates were identified under the genus *Acidovorax*, *Brevundimonas* and *Stenotrophomonas*. These genera, emended from the genus *Pseudomonas* have not been widely reported from seafood sources and further investigation should be carried out to ascertain their role in spoilage microflora.

- Finally, Sherlock MIS has proved to be an effective rapid technique for identification of bacteria associated with seafood spoilage.